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MODELING HUMAN GERM CELL DEVELOPMENT WITH PLURIPOTENT STEM CELLS AND CHARACTERIZING THE PUTATIVE OOGONIAL STEM CELLS

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Cover: Meiotic cell from Cynomolgus Monkey testis: SCP1 (red), Centromere protein A (green), DAPI (blue).

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For my husband

ABSTRACT

The current understanding of human germ cell development is limited and is at most parts extrapolated from studies of mice or other model organisms. Particularly, the specification events of human germ cell lineage are largely unknown, which is at least in part due to inaccessibility of early stages of human development. In addition, debate on the existence of oogonial stem cells (OSCs) in adult ovaries further indicate that more research is needed in order to elucidate human germ cell development.

Human pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), offer a promising *in vitro* strategy to study fundamental questions regarding human germ cell development and to model genetically caused infertility. In this thesis, we used human PSCs to elucidate the genetic requirements of human germ cell development. Specifically, we assessed the potential of human iPSCs to differentiate to pre-meiotic and post-meiotic germ cells, studied the function of germ cell specific NANOS3 and Deleted in azoospermia like (DAZL) proteins via over expression in hESCs, and optimized clinically relevant iPSC reprogramming conditions for the derivation of patient specific iPSCs from fibroblasts of infertile Klinefelter syndrome (KS) patients. Furthermore, we studied the existence of OSCs by isolating and characterizing cells from adult human and mouse ovaries.

We found that human iPSCs derived from fetal- and adult somatic cells could differentiate to pre-meiotic germ cells with similar or higher efficiency relative to hESCs. Furthermore, in response to the over expression of intrinsic meiotic regulators, we observed that like hESCs, iPSCs formed meiotic and post-meiotic haploid cells. In addition, we identified several candidate genes for NANOS3 and DAZL mediated functions by over expression in hESCs. We found that human NANOS3 may have a conserved role in suppression of differentiation and inhibition of apoptosis, and DAZL in induction of germ cell differentiation and cell cycle arrest. Furthermore, we identified a possible new role of DAZL in inhibition of cell migration. For optimizing clinically relevant iPSC reprogramming conditions, we achieved efficient reprogramming using xeno-free and chemically defined laminin-521 and NutriStem medium, and derived KS patient specific iPSCs. Finally, we contradicted the previous study of OSCs by showing that the reported DEAD (Asp-Glu-Ala-Asp)-box polypeptide 4 (DDX4) antibody –based isolation of OSCs did not select for DDX4 expressing cells from adult human or mouse ovaries, and the isolated cells were not functional OSCs.

In conclusion, human PSCs have an enormous potential to elucidate the complex mechanisms of human germ cell development, and may ultimately contribute to new strategies for the diagnosis and treatment of infertility in years to come. In addition, we found no evidence for the existence of OSCs in adult human ovaries, thus, supporting the central dogma of fixed ovarian reserve.

LIST OF SCIENTIFIC PAPERS

- I. **Sarita Panula**, Jose V. Medrano, Kehkooi Kee, Rosita Bergström, Ha Nam Nguyen, Blake Byers, Kitchener D. Wilson, Joseph C. Wu, Carlos Simon, Outi Hovatta and Renee A. Reijo Pera.
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LIST OF ABBREVIATIONS

1N	Haploid cell
2i	Mitogen-activated protein kinase kinase inhibitor and glycogen synthetase kinase 3 inhibitor
2me	2-mercaptoethanol
α -MEM	Minimum essential medium, alpha
ANOVA	Analysis of variance
AP	Alkaline phosphatase
AP2 γ	Transcription factor AP-2 gamma
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
C-MYC	v-Myc myelocytomatosis avian viral oncogene homolog
CAG	CMV early enhancer/chicken beta actin
Ct	Threshold cycle
CXCL5	Chemokine (C-F-C motif) ligand 5
CYP26B1	Cytochrome P450, family 26, subfamily B, polypeptide 1
DAPI	4',6'-Diamidino-2-phenylindole
DAZ	Deleted in azoospermia
DAZL	Deleted in azoospermia like
DDX4	DEAD (Asp-Glu-Ala-Asp)-box polypeptide 4
DMEM	Dulbecco's modified eagle medium
DPPA3	Developmental pluripotency associated 3
DSB	Double strand break
E	Embryonic day
EB	Embryoid body
EGFP	Enhanced green fluorescent protein
EpiSC	Epiblast stem cell
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FAM110C	Family with sequence similarity 110, member C

FBS	Fetal bovine serum
FGF9	Fibroblast growth factor 9
FISH	Fluorescence <i>in situ</i> hybridization
FOXL2	Forkhead box L2
FSH	Follicle stimulating hormone
GABRP	Gamma-aminobutyric acid A receptor, pi
GDF9	Growth differentiation factor 9
GDNF	Glial cell line derived neurotrophic factor
GSC	Germline stem cell
GSK3	Glycogen synthetase kinase 3
HDF	Human dermal fibroblast
HE	Hematoxylin and eosin
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
hESC	Human embryonic stem cell
HIP1R	Huntingtin interactin protein 1 related
HRP	Horseradish peroxidase
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IFITM1	Interferon induced transmembrane protein 1
IFITM3	Interferon induced transmembrane protein 3
iPSC	Induced pluripotent stem cell
ISYNA1	Inositol-3-phosphate synthase 1
IVF	<i>In vitro</i> fertilization
JAG2	Jagged 2
kb	Kilobase
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KITLG	KIT ligand
KLF4	Krüppel-like factor 4
KS	Klinefelter syndrome
KSR	Knockout serum replacement
LCP1	Lymphocyte cytosolic protein 1

LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
MEF	Mouse embryonic fibroblast
MEK	Mitogen-activated protein kinase kinase
mESC	Mouse embryonic stem cell
mTmG	Membrane-targeted Tomato and Green
NEAA	Non-essential amino acids
OLFM2	Olfactomedin 2
orf	Open reading frame
OSC	Oogonial stem cell
PAX6	Paired box 6
Penstrep	Penicillin-streptomycin
PGC	Primordial germ cell
PGCLC	Primordial germ cell like cell
PIDD1	p53-induced death domain protein
PLZF	Promyelotic leukemia zinc finger ortholog
POU5F1	POU class 5 homeobox 1
PRDM1	PR domain containing 1
PRDM14	PR domain containing 14
PRKCSH	Protein kinase C substrate 80K-H
PSC	Pluripotent stem cell
RA	Retinoic acid
RBP	RNA-binding protein
RIPA	Radio-immunoprecipitation assay
ROCKi	Rho-associated coiled-coil kinase inhibitor
RPRM	Reprimo
RT	Reverse transcription
SC	Synaptonemal complex
SCID	Severe combined immunodeficient
SCP	Synaptonemal complex protein
SNL	Soriano ES cell feeder cell line SNL 76/7 STO cells

SOX	Sex determining region Y-box
SRY	Sex determining region Y
SSC	Spermatogonial stem cell
SSEA	Stage-specific embryonic antigen
STRA8	Stimulated by retinoic acid gene 8
TGF β	Transforming growth factor beta
TRA	Tumor-related antigen
WNT	Wingless type MMTV integration site family
XCI	X chromosome inactivation
XIST	X specific transcript
ZP	Zona pellucida

1 INTRODUCTION

Germ cell development has been comprehensively studied in mice, but very little is known in the human. Particularly, the molecular and cellular events leading to specification of the germ cell lineage in embryonic development are largely unknown due to the inaccessibility of early human embryos. Although spermatogenesis and oogenesis, occurring in adulthood, are more extensively studied, our knowledge on the maintenance of spermatogenesis by a stem cell population is still limited. Furthermore, recently emerged debates on the existence of oogonial stem cells (OSCs) in adult mammalian ovaries show that more research is needed to examine the specifics of human germ cell development.

Human pluripotent stem cells (PSCs) offer a great strategy to study fundamental questions regarding human germ cell development, including the molecular pathways and genetic requirements for germ cell formation. In addition, the revolutionary technology of induced pluripotent stem cell (iPSCs) reprogramming enables the derivation of patient specific PSCs providing an *in vitro* platform to model infertility, and in years to come may lead to a new form of assisted reproduction.

1.1 PLURIPOTENT STEM CELLS

PSCs are undifferentiated cells that are capable for unlimited self-renewal and differentiation into any type of cell representing the three embryonic germ layers: endoderm, mesoderm and ectoderm, as well as the germ line. These specific characteristics make PSCs a powerful tool to study cell lineage commitment and early development. Furthermore, PSCs have a great potential to be used as a treatment for degenerative diseases. In fact, two clinical trials using PSCs as a treatment are currently ongoing for spinal cord injury and age-related macular degeneration (1, 2).

1.1.1 Human embryonic stem cells

Human embryonic stem cells (hESCs) are PSCs that are derived from left over or poor quality embryos produced by *in vitro* fertilization (IVF) for clinical purposes (Figure 1). The inner cell mass (ICM) of a blastocyst stage embryo is isolated and placed into culture conditions that support undifferentiated cell proliferation, resulting in the derivation of a stable hESC line (3). Derivation of the first hESC lines was reported in 1998, and ten years after, over one thousand new hESC lines had already been described (4). The derivation technique and culture conditions have been improved over the years, and now it is possible to derive hESCs also from a single blastomere of a morula (8-10 cell stage embryo), without the need to destroy the embryo (5, 6).

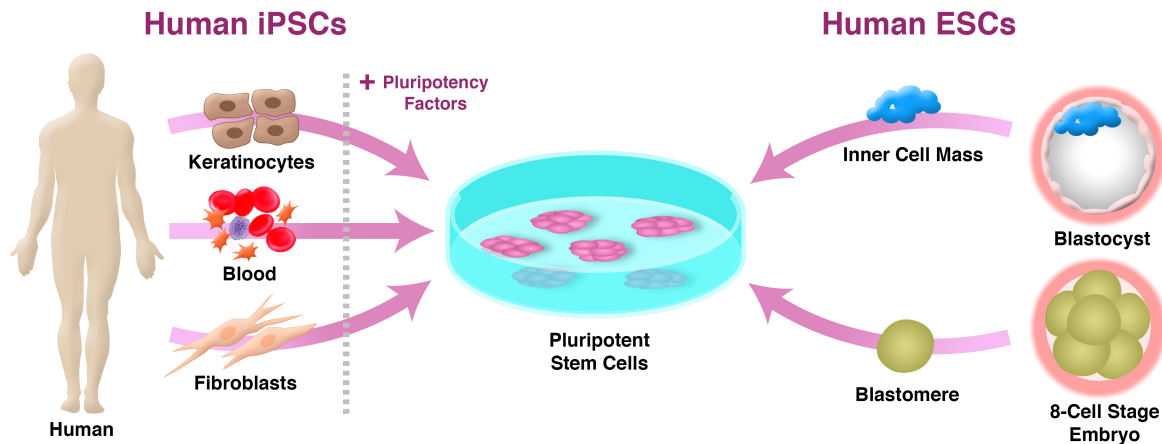


Figure 1. Human pluripotent stem cells. Various types of human somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) by transient expression of pluripotency factors. Human embryonic stem cells (ESCs) can be derived from the inner cell mass of a blastocyst stage embryo or from a single blastomere of 8-cell stage embryo.

1.1.1.1 Characterization

The characteristic morphology of hESCs is a high nucleus to cytoplasmic ratio and prominent nucleoli, and they form relatively flat, compact colonies with distinct cell borders (3). hESCs can be cultured for extended time periods, while retaining their pluripotency and normal karyotype. The criteria to assess pluripotency include a set of cell surface markers and transcription factors, and the capacity to differentiate *in vitro* and *in vivo* (7).

Standard cell surface markers that are expressed by hESCs, include stage-specific embryonic antigen (SSEA) 3, SSEA4, tumor-related antigen (TRA)-1-60, TRA-1-81 and alkaline phosphatase (AP) (3). The expression of other stem cell related cell surface markers has also been described for hESCs, such as CD9 and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) (8). Core transcription factors POU class 5 homeobox 1 (POU5F1, also known as OCT4), NANOG and SRY (sex determining region Y)-box (SOX) 2 are required for pluripotency and self-renewal of hESCs, and are routinely used in the characterization of hESCs (9).

The potential of hESCs to differentiate towards the three germ layers is analyzed by *in vitro* and *in vivo* assays. Upon injection into severe combined immunodeficient (SCID) mice, hESCs form teratomas, which contain cells of endodermal, ectodermal and mesodermal origin (3). A comparable *in vitro* assay is the formation of cell aggregates called embryoid bodies (EBs), which contain spontaneously differentiated cells from all three germ layers (10). These two methods are most commonly used for the characterization of new hESC lines, however, for using hESCs as a model for developmental studies, directed differentiation to a specific cell lineage is preferred. Efficient differentiation protocols for obtaining e.g. midbrain dopamine neurons, hepatocytes, and cardiomyocytes have been reported (11-13). The differentiation of PSCs towards the germ line is discussed in section 1.6.

1.1.1.2 Culture conditions

The first hESC lines were derived on mitotically inactivated mouse embryonic fibroblasts (MEFs) as feeder cells, and using culture medium containing fetal bovine serum (FBS) (3). These undefined culture conditions, however, create variability and expose hESCs to xenogeneic components. For possible clinical use of hESCs or their derivatives, good manufacturing practice should be followed, including defined culture components and minimal exposure to animal-derived substances (14).

The animal-derived MEFs can be substituted with human cells, and FBS with knockout serum replacement (KSR) and basic fibroblast growth factor (bFGF), although KSR is not completely devoid of animal substances (15-17). In the absence of serum, bFGF is needed for the undifferentiated self-renewal of hESCs, and it has now been well established that bFGF signaling is required for hESC maintenance (18).

For the establishment of feeder-free cultures, several matrix proteins have been used for coating of culture plates. Matrigel, a mouse tumor extract containing a mixture of proteins, is most commonly used with either a conditioned medium collected from MEF culture plates or commercially available mTeSR1 medium containing a high concentration of bFGF (19, 20). Xeno-free derivatives of the mTeSR1 medium have been used to establish completely xeno-free and feeder-free culture conditions, using either a mixture of collagen IV, vimentin, fibronectin and human placenta laminin, or human recombinant laminin-511 alone (20, 21).

Passaging of hESCs has conventionally been done in small clusters of cells either manually or enzymatically (3, 16). The poor survival of hESCs after dissociation into single cells has limited the manipulation of the cells, however, Rho-associated coiled-coil kinase inhibitor (ROCKi) was found to increase the cell survival of dissociated hESCs and facilitated the use of hESCs in several applications, including gene transfer and cell sorting (22, 23). Recently, human recombinant laminin-521 together with chemically defined and xeno-free medium NutriStem were shown to support the culture of single-cell dissociated hESCs without the use of ROCKi, and furthermore, laminin-521/E-cadherin matrix allowed clonal derivation of hESCs from a single blastomere (5).

1.1.2 Human induced pluripotent stem cells

The generation of induced pluripotent stem cells (iPSCs) from mouse embryonic and adult fibroblasts was first described in 2006 (24). Takahashi *et al.* demonstrated that forced expression of specific transcription factors reprogrammed adult fibroblasts into iPSCs that were remarkably similar to ESCs in gene expression profile, self-renewal and differentiation potential. In an elegant screen of 24 candidate genes, a set of four factors was identified essential for the generation of iPSCs: *Pou5f1*, *Sox2*, Krueppel-like factor 4 (*Klf4*), and v-Myc myelocytomatosis avian viral oncogene homolog (*c-Myc*). Shortly after, these same factors were used for the derivation of human iPSCs (25). Around the same time, another group reported independently the use of *POU5F1*, *SOX2*, *NANOG* and *LIN28* for human iPSC

reprogramming (26). In a short amount of time, numerous studies aimed at optimizing iPSC generation from various somatic cells have been published (Figure 1) (27).

1.1.2.1 Methods for iPSC generation

The original methods for human iPSC induction used retroviral or lentiviral vectors for gene delivery; however, this results in integration of the transgenes into the cell genome with risk of genomic instability and chromosomal aberrations (25, 26). In addition, the derivation and culture conditions were dependent on the use of mouse feeder cells. Several improvements for the reprogramming conditions have now been reported, in terms of higher efficiency, integration-free delivery, and feeder-free conditions.

Non-integrating virus, such as Sendai virus, has been used for efficient reprogramming of iPSCs (28). The Sendai virus vectors are gradually depleted from the cells through several cell divisions, which result in transgene-free iPSCs. A similar strategy is deployed when using non-integrating episomal plasmids, which additionally eliminates the need for handling viruses (29, 30). In addition to the vector-based methods, direct transfection with mRNAs or proteins has also been reported, although these methods are labor intensive and are not commonly used (31, 32).

Although the majority of iPSC reprogramming conditions use mouse feeder cells, some feeder-free reprogramming methods based on various matrices, including Matrigel and vitronectin, have been reported (33-36). These methods often result in less efficient reprogramming or are used together with transgene integrating methods. Reprogramming with Sendai virus and synthetic mRNAs in feeder-free conditions has been successful, however, the conditions were not xeno-free (37, 38). Only recently, iPSC reprogramming with episomal plasmids in feeder-free and xeno-free conditions using laminin-511 E8 fragment and StemFit medium was reported; although this was inefficient and not completely chemically defined (39). Thus, combining an integration-free method with chemically defined, xeno-free and feeder-free condition is still a challenge.

1.1.3 Primed and naïve pluripotent stem cells

Human and mouse PSCs, including ESCs and iPSCs, have distinct molecular and biological characteristics. Human PSCs grow as flat colonies and require bFGF and transforming growth factor beta (TGF β) for self-renewal, whereas mouse PSCs form compact dome-like colonies, depend on leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) 4 to proliferate, and have high clonal efficiency (40). Although both human and mouse PSCs express the core pluripotency genes, there are also differences in their expression profiles, e.g SSEA1 is expressed in mouse PSCs but not in human (3, 41, 42). Mouse epiblast stem cells (EpiSCs) derived from post-implantation epiblast, however, resemble human PSCs more closely in the culture requirements (43). Thus, it has been suggested that two distinct phases of pluripotency exist, with mouse PSCs representing a naïve state and mouse EpiSC and human PSCs representing a primed state (44).

1.1.3.1 Conversion from primed to naïve

The naïve state of mouse PSCs can be stabilized by using dual inhibition of mitogen-activated protein kinase kinase (MEK) and glycogen synthase kinase 3 (GSK3) together with leukemia inhibitory factor (LIF) (45). Further, EpiSCs can be converted to mouse ESC-like naïve state by using chemical compounds, including the MEK and GSK3 inhibitors (2i), alone or together with over expression of reprogramming genes, such as *Klf4* (46-49). Based on these findings, human PSCs were converted into naïve-like cells using 2i/LIF together with genetic manipulation (50, 51). Recently, several groups have reported culture conditions based solely on chemical compounds that can support the establishment of naïve-like human PSCs (52-56). Each group used a different chemical cocktail and basal medium, resulting in substantial differences between the naïve-like cells from different groups (56). Although the transcriptional and epigenomic profiles of naïve-like human PSCs closely resembled the naïve mouse pluripotency, some differences remained, e.g. in X chromosome inactivation (XCI) (56). However, it is still unclear how naïve human pluripotency should be defined and examined.

1.1.3.2 X chromosome inactivation

In female mammalian cells, one copy of the two X chromosomes is inactivated in order to achieve dosage compensation to balance gene expression levels. XCI is an epigenetic process that is regulated by X inactive specific transcript (*Xist*), a long noncoding RNA that coats the X chromosome and triggers the silencing effect (57). In mouse embryos, XCI is first seen in the paternal X chromosome, which is subsequently reactivated in the preimplantation epiblast, thus, mouse ESCs derived from these cells have two active X chromosomes (40). In postimplantation epiblast, random XCI occurs and EpiSCs derived from these cells have XCI (40). Therefore, XCI status of mouse PSCs has been used to define naïve and primed states. Of note, XCI is erased again specifically in primordial germ cells (PGCs) during mouse embryonic development (58).

The status of XCI in human embryos and PSCs is not as clear. Paternal XCI does not occur in early human embryo and *XIST* expression is seen in both X chromosomes of the ICM cells, despite their active status (59). hESCs derived from the ICM, can have active X chromosomes or XCI with or without *XIST* coating (40). Similarly, differences in the XCI of the human naïve-like cells were reported, even increased *XIST* expression was observed after the conversion to naïve-like cells (56). Taken these differences, active X chromosomes might not be a defining feature of naïve pluripotency in the human.

1.2 GERM CELL DEVELOPMENT

Germ cells are unique cells with a sole function to transmit genetic information between generations and thus, secure the survival of a species. Germ cell development is a complex process that starts early in embryonic development and continues through the reproductive life of the individual, resulting in mature sperm or oocytes. Little is known about the early germ cell development in human, due to the inaccessibility of the embryo and the scarcity of

the germ cell population at that time. Therefore, most of our knowledge is extrapolated from studies with mice. Continuous research is done in order to obtain detailed information on the molecular and cellular mechanisms controlling the fate of the germ cells throughout the long process of germ cell development.

1.2.1 Specification and migration

The specification events of human germ cells are unknown, as the studies would require embryonic day (E) 9–16 embryos and are therefore impractical. These events, however, are studied in detail in mice. Mouse germ cell precursors are specified on E6.25 from the proximal epiblast cells in response to BMPs from the extraembryonic tissues (60, 61). These few precursors generate a founder population of about 40 PGCs that are identified as AP positive cells in the posterior primitive streak in the extraembryonic mesoderm at E7.25 (62, 63). At E8.0, PGCs are residing in the yolk sac mesoderm, close to the junction with the allantois (64). The corresponding developmental age of human embryos is 3–4 weeks, and the earliest human PGCs have been identified in the same location as mouse PGCs (65, 66). Similar to mouse, human PGCs were characterized as large round cells with large nucleus and positive for AP. At E8.5 for mouse and 5 weeks of development for human, the PGCs initiate their migration through the hindgut and its mesentery into the genital ridges. By E11.5 for mouse and 6 weeks of development for human, most of the PGCs have arrived at their destination and colonized the developing gonads (Figure 2) (65-67).

The migration of PGCs seems to be a complex process, depending on passive and active events, appropriate physical pathway and directional cues. In addition, the migratory PGCs must survive and proliferate to increase in number (66). Although the exact process of PGC migration is not known, the initial translocation of PGCs from the yolk sac into the hindgut epithelium is believed to be passive, aided by the morphogenetic rearrangements of the embryo (68). After this, PGCs may be capable for active migration, shown by the change in morphology from round cells into an irregular shape with protrusions and pseudopodia (65). The migration may be mediated by a chemotactic factor, KIT ligand (KITLG), which is expressed by the somatic cells along the migratory pathway, while KIT is expressed in the PGCs (69, 70). This interaction may also have a critical role in the survival of PGCs, as down regulation of KITLG in extragonadal areas has been shown to promote apoptosis in mouse PGCs that failed to migrate correctly (71). Furthermore, apoptosis in mouse PGCs is mediated by the pro-apoptotic gene BCL2-associated X protein, which has been shown to act downstream of KIT (72). KITLG is expressed also by the autonomic nerve fibers of the dorsal mesentery, and human PGCs have been shown to be in close contact with them (73). Thus, it has been proposed that from 6 weeks of development, human PGCs travel along the developing nerve fibers into the gonads (70). However, further studies are needed to elucidate the complex mechanism of migration, survival and proliferation of PGCs.

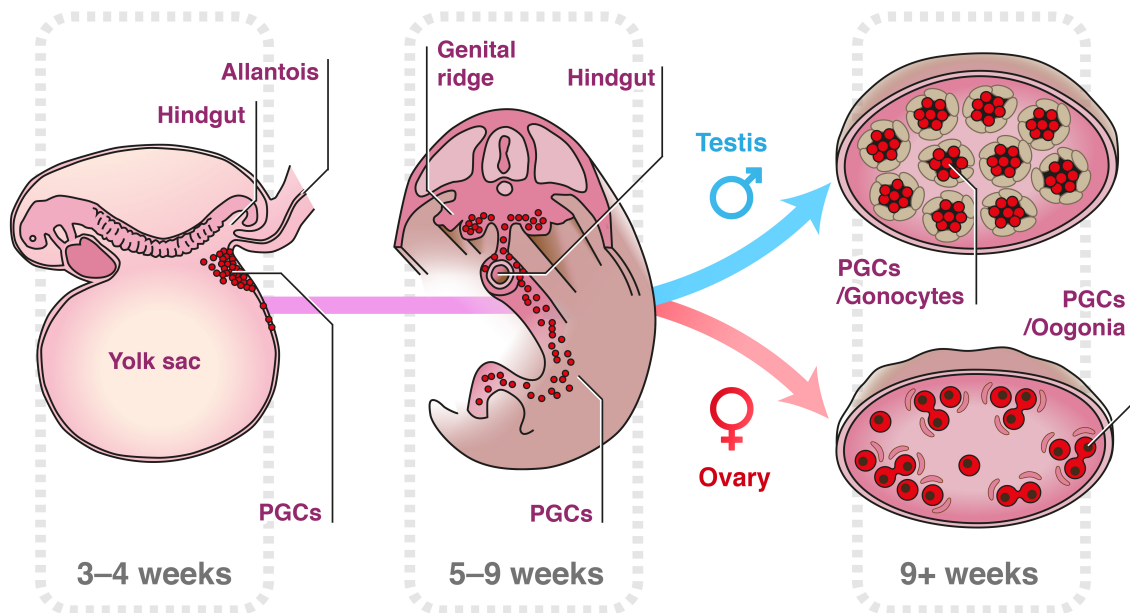


Figure 2. Human primordial germ cell (PGC) development. Around 3–4 weeks of embryonic development, PGCs are residing in the yolk sac mesoderm, close to the junction with the allantois. Between 5–9 weeks of development, PGCs proliferate and migrate through the hindgut and its mesentery into the genital ridges and colonize the developing gonads. Around 9 weeks of development, PGCs start to differentiate to either oogonia or gonocytes depending on the gonadal sex. In female gonads, oogonia undergo mitotic proliferation forming clusters of cells that are connected by intercellular bridges and surrounded loosely by somatic cells. Around 10–12 weeks of development, oogonia start to enter into meiosis, arresting at the end of the prophase I and become primary oocytes. In male gonads, gonocytes become enclosed within the testis cords and are mitotically active until 5 months of development, followed by decreased proliferation period for the duration of the fetal development.

1.2.2 Colonization and sex determination

Human PGCs proliferate during migration, but mostly after reaching the gonads, resulting in about 450,000 germ cells in female and 150,000 in male by 9 weeks of development (74). The factors regulating human PGC proliferation are largely unknown, although BMP4, LIF, bFGF and forskolin have promoted *in vitro* proliferation of human PGCs (75, 76). The PGCs maintain their bipotentiality until sex specific differences start to occur. The fate of the germ cells to form either oocytes or sperm is independent of the germ cell sex, but rather influenced by environmental cues from the fetal gonads (77).

1.2.2.1 Gonadal sex determination

Differentiation of the somatic cells in an XY gonad is induced by the expression of the Y chromosome linked gene, *SRY*, which upregulates SOX9 expression (78). The expression induces cellular and morphological changes, including the differentiation of Sertoli and Leydig cells, and the formation of testis cords; which are the precursors of the seminiferous tubules around 7–9 weeks of development. The morphological changes in an XX gonad are less profound and the molecular genetics involved are poorly understood, however, wingle-type MMTV integration site family, member (*WNT*) 4 and R-spondin 1 pathway along with forkhead box L2 (*FOXL2*) expression have been associated with ovarian development (78).

In the ovary, germ cell cords are visible at 6 weeks of development, but become fragmented and pushed towards the periphery, resulting in the formation of medulla and a germ cell-rich cortex by 13 weeks of development.

1.2.2.2 Oogonia

After reaching the developing gonads, PGCs differentiate to either oogonia or gonocytes depending on the gonadal sex. Oogonia are formed around 9 weeks of development and undergo mitotic proliferation for several weeks, mostly forming clusters of cells that are connected by intercellular bridges and surrounded loosely by somatic cells (74, 79). Around 10–12 weeks of development, oogonia start to enter into meiosis, arresting at the end of the prophase I and become primary oocytes (80). The period of mitotic proliferation and meiosis is overlapping, and a heterogeneous germ cell population is observed even until the fifth month of development, with meiotic entry mostly in the inner cortex and mitotic proliferation in the outer cortex (78, 81). An estimate of 7×10^6 oocytes is formed during this time (81, 82). However, many of the oocytes in the nests undergo apoptosis during the follicle assembly that begins around 18 weeks of development and continues into the third trimester (79, 81, 82). The oocyte nests are broken down and single oocyte becomes surrounded by pregranulosa cells, forming a primordial follicle. The primordial follicles constitute a pool of around 3×10^5 oocytes at birth, with most remaining quiescent for several years (83).

1.2.2.3 Gonocytes

PGCs in the male gonad become enclosed within the testis cords and are called gonocytes thereafter. Gonocytes are mitotically active with the highest rates of proliferation observed between the third and fifth months of development, followed by decreased proliferation period for the duration of the fetal development, and a small increase in proliferation at 2.5–6 months after birth (84). Gonocytes represent a heterogeneous cell population with varying expression profiles and can be divided further into three subpopulations: mitotic, and transitional 1 and 2 prespermatogonia (85). Around the second trimester, most but not all mitotic prespermatogonia start differentiating to transitional spermatogonia, which downregulate the expression of pluripotency markers and become mitotically quiescent (85). Gonocyte migration from the center of the cords to the basement membrane seems to be a prerequisite for their differentiation and has been proposed to be mediated by a transient KIT expression in translocating gonocytes, at least in rodents (85). The transitional 1 prespermatogonia are mostly found in pairs, and transitional 2 in groups of cells interconnected through cytoplasmic bridges, similar to differentiating oogonia (74). Transitional 2 prespermatogonia that colonize the basement membrane are believed to establish the pool of spermatogonial stem cells (SSC) that maintain spermatogenesis in adulthood (85).

1.2.2.4 Germ cell sex determination

The molecular mechanism behind the control of sex-specific entry into meiosis has been extensively studied in mice. Two secreted molecules, retinoic acid (RA) and fibroblast

growth factor 9 (FGF9), seem to have key roles in this event (77). In the fetal ovary, RA induces the expression of stimulated by retinoic acid gene 8 (*Stras8*) in oogonia, and triggers their entry into meiosis. In the fetal testis, RA is actively degraded by cytochrome P450, family 26, subfamily b, polypeptide 1 (CYP26B1) resulting in low *Stras8* levels and no meiotic entry. In addition, FGF9, produced by Sertoli cells in the fetal testis, has been shown to inhibit meiosis and maintain the expression of pluripotency markers (77). In humans, RA and STRA8 seem to be involved in the meiotic regulation in the fetal ovary; however, CYP26B1 function in fetal testis did not seem to be conserved (80, 86). Furthermore, factors triggering the mitotic arrest of prespermatogonia are still unknown for both human and mouse, although FGF9 has been suggested to have a role (77).

1.2.3 Oogenesis and spermatogenesis

1.2.3.1 Oogenesis

Primordial follicles are recruited into the growing follicle pool continuously, just after follicle formation and until menopause. The recruitment rate of primordial follicles increases from birth until 14 years of age, when about 900 follicles are recruited every month, followed by slow decline in numbers with age (83). When less than about 1000 primordial follicles are left in the ovary, follicle development stops and women undergo menopause, at around 50 years of age (83).

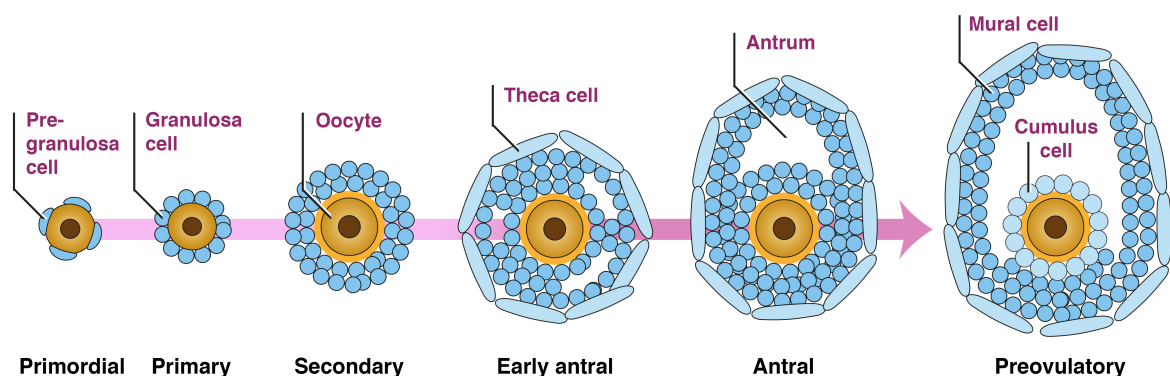


Figure 3. Folliculogenesis. Primordial follicle, consisting of a single oocyte surrounded by pregranulosa cells, develops into primary follicle with differentiated granulosa cells. During secondary follicle development, multiple layers of granulosa cells are formed, and oocyte starts to grow in size and forms a surrounding zona pellucida layer. Theca cells surround the follicle while a fluid filled cavity (antrum) appears at early antral and antral stages of follicle development. At preovulatory stage, the outermost layer of granulosa cells is called mural cells, and specialized granulosa cells, called cumulus cells, surround the oocyte, which is now matured and competent to resume meiosis.

The primordial follicle, containing a single oocyte, develops through primary and secondary stages into antral follicle, but at this stage, most of the follicles undergo atretic degeneration (87). It is not until puberty, when a group of about 10 antral follicles escape apoptosis via follicle stimulating hormone (FSH) signaling each month, and one dominant follicle continues to grow until preovulatory follicle, finally resulting in the ovulation of a matured oocyte competent for fertilization (Figure 3) (87). The entire growth phase from primordial

follicle to preovulatory follicle takes longer than 8 months, and the follicle grows in size from about 30 μm to 20 mm (87, 88). The longest time is taken in the primordial to primary, and primary to secondary follicle development, with more than 120 days spent on both steps, although the biggest growth in follicle size is observed after the antral follicle formation (87).

During the follicle development, the oocyte grows in size and matures. The process is regulated by endocrine and paracrine factors, and junctional interactions with the surrounding somatic cells in the follicle (89). The oocyte itself seems to be largely orchestrating the follicle maturation, and at least two oocyte-secreted factors, growth differentiation factor 9 (GDF9) and BMP15, have been directly implicated in the process (89). At the secondary follicle stage, oocyte grows in size, and has a huge rise in RNA and protein synthesis and in the number of cell organelles, accumulating molecules and organelles that are required for the development of the preimplantation embryo (88). In addition, the formation of zona pellucida (ZP) around the oocyte is observed at this stage, consisting of glycoproteins secreted by the oocyte, ZP1, ZP2 and ZP3. At the end of the antral follicle stage, oocyte has reached its final size of about 120 μm , and has become competent to resume meiosis.

Once the primary oocyte, still arrested at meiosis I, has grown and matured, it resumes meiosis in response to luteinizing hormone (LH) surge (89). The chromosomes condense and are divided into two cells in asymmetric cell division, resulting in a large oocyte and a small polar body, which eventually degenerates (89). The meiotic maturation is complete upon arrest at metaphase of meiosis II, forming a secondary oocyte. Meiosis resumes again only after fertilization by sperm, resulting in the formation of second polar body and an oocyte with a haploid chromosome content, and the initiation of embryo development (88, 89).

1.2.3.2 Spermatogenesis

Spermatogenesis starts in early puberty and consists of cycles where SSCs or undifferentiated spermatogonia undergo mitotic and meiotic divisions and differentiate into spermatids. From each SSC, up to 16 spermatids are formed in a process lasting 74 days, which results in a highly efficient system producing an average of 60 million sperm per ml of ejaculate (90, 91). Although daily sperm production tends to decline with advancing age, sperm production continues throughout the adult male life (90).

Spermatogenesis occurs within the seminiferous tubules, which consist of Sertoli cells and germ cells at different stages, surrounded by peritubular cells (Figure 4). Spermatogenesis is heavily dependent on gonadotrophins, which affect the germ cells through the somatic cells found in the testis (92). LH induces the Leydig cells, located in the interstitial space, to produce testosterone, and Sertoli cells express receptors for both testosterone and FSH. Sertoli cells are the only somatic cells in the tubules and have several important roles in spermatogenesis. They nurture and maintain cellular associations with germ cells throughout the process of spermatogenesis, until the controlled release of mature spermatids into the tubular lumen (93). Sertoli cells also secrete several proteins and growth factors, such as glial

cell line derived neurotrophic factor (GDNF) and bFGF, which have been shown to promote SSC proliferation, and RA, which induces meiosis (94).

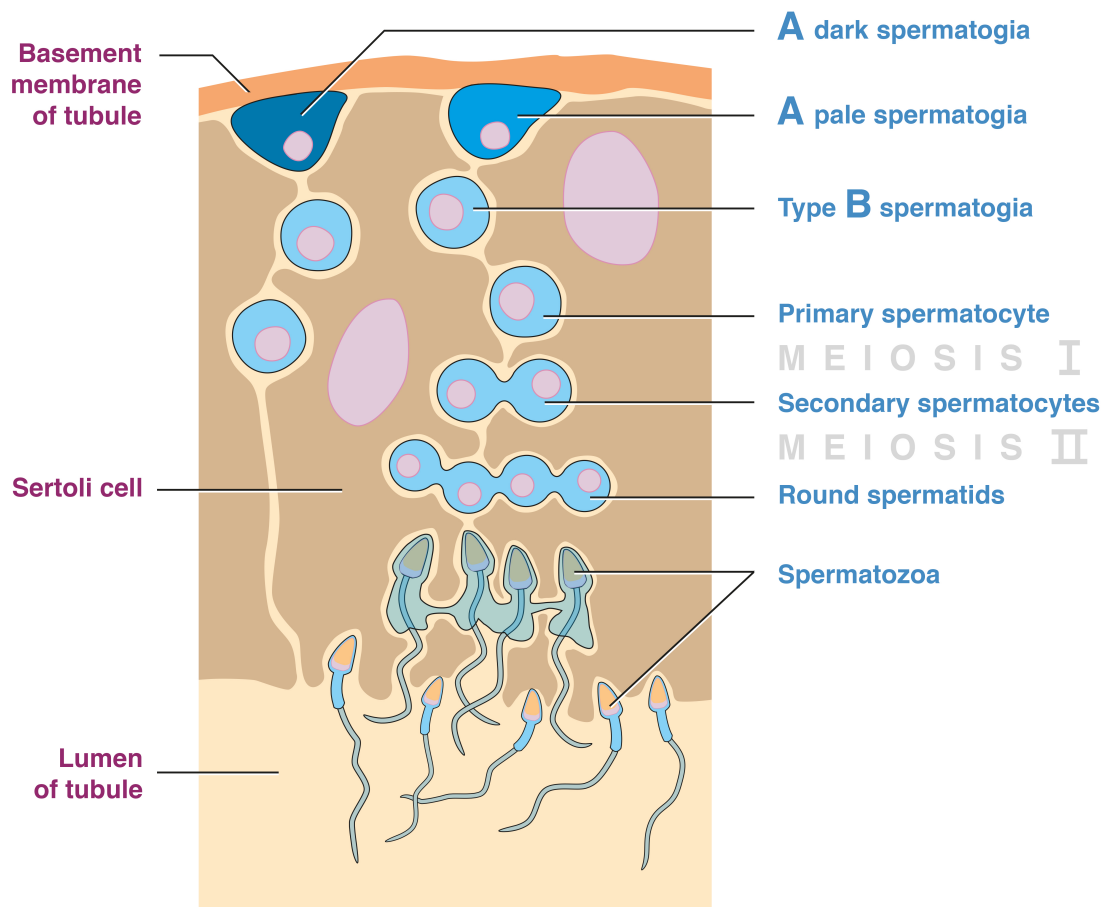


Figure 4. Spermatogenesis. Spermatogenesis occurs within the seminiferous tubules, which consist of Sertoli cells and germ cells at different stages and a fluid filled lumen, surrounded by peritubular cells. A_{dark} and A_{pale} spermatogonia are located on the basement membrane of the tubule. A_{pale} spermatogonia differentiate to type B spermatogonia, which divides by mitosis forming primary spermatocytes that enter into meiosis. Primary spermatocytes, connected by intercellular bridges, grow in size and move toward the lumen, forming secondary spermatocytes after completion of meiosis I. Secondary spermatocytes are smaller in size and proceed through meiosis II, forming haploid round spermatids, which are then transformed into spermatozoa and released into the lumen.

Undifferentiated spermatogonia are semicircular in shape and are located on the basement membrane of the seminiferous tubules. They can be divided into two groups, named A_{dark} and A_{pale} based on the staining intensity with hematoxylin (95). A_{dark} spermatogonia are relatively quiescent, while A_{pale} are mitotically active and differentiate to type B spermatogonia. The A_{dark} and A_{pale} spermatogonia are considered to represent reserve and active SSCs, respectively, although the identity of human SSCs is not well established (95). Type B spermatogonia divide by mitosis forming two cells that enter into meiosis, now called primary spermatocytes (93). Once the primary spermatocytes progress through the prophase I stages of meiosis I, they become larger in size and move towards the lumen. Secondary spermatocytes are formed after completion of meiosis I, are smaller in size and go directly into meiosis II, forming haploid round spermatids (93). During the mitotic and meiotic

divisions, the daughter cells stay bound together through intercellular cytoplasmic bridges, enabling highly synchronized events (90).

In the following several weeks, the round spermatids are transformed into spermatozoa in a process called spermiogenesis, which is actively guided by the Sertoli cells and resulting in the release of maturing spermatozoa into the lumen (90). The spermatids undergo a dramatic decrease in size by chromatin and nuclear condensation and reduction in the volume of cytoplasm, and change in morphology by acquiring an acrosome cap and a tail (93). Once the spermatozoa are released to the tubule lumen, they progress through the epididymis and mature to functional sperm able to move and fertilize an oocyte (96).

1.3 GERM CELL RELATED GENES

The characterization and identification of germ cells at different developmental stages is heavily relying on protein expression analysis. In mice, the germ cell development is relatively synchronized, whereas in humans, a more heterogeneous cell population is observed during fetal development that can only be identified through molecular markers. The expression pattern of commonly used markers for germ cells at different stages is discussed in the following section. In addition, the known functions of genes required for mouse germ cell development are described and compared to human germ cells.

1.3.1 Specification genes

The key regulators for mouse PGC specification, starting at E6.25, are identified as PR domain containing 1 (*Prdm1*, also known as *Blimp1*) and PR domain containing 14 (*Prdm14*) that are induced by BMP4 (97). PRDM1 induces the expression of transcription factor AP-2 gamma (*AP2γ*) at E6.75, and together these three genes are required for PGC specification in mice (98). The expression of interferon induced transmembrane protein 3 (IFITM3) is also induced by BMP4 at E6.25 in the germ cell precursors, with increased expression at E7.0 in the PGCs relative to surrounding somatic cells, however, IFITM3 is suggested to aid early migration of PGCs rather than their specification (99-101). At E7.0, a germ cell specific expression of developmental pluripotency associated 3 (*Dppa3*, also known as *Stella*) is induced by PRDM1 and PRDM14, and it is often used as a first defining marker of specified PGCs (98, 99).

PRDM1, PRDM14 and AP2γ expression in the mouse PGCs are continued during migration, until differentiation to gonocytes (102-104). The expression of these markers in early human PGCs is unknown, however, PRDM1 and AP2γ are reported to be expressed in PGCs/gonocytes at around 12–22 weeks of human development (105-107). Thus, suggesting that these genes may have a conserved role in human PGC specification. The expression of PRDM14 in human germ cells is not studied, however, it is highly expressed in hESCs and suggested to be involved in the maintenance of pluripotency by suppressing differentiation (108).

1.3.2 Pluripotency genes

During mouse embryonic development, pluripotency-associated genes are expressed in the ICM and in the epiblast, but are subsequently restricted to the early germ cell lineage. *Pou5f1* expression is restricted to mouse PGCs from around E7.5 onwards and the expression is maintained until the onset of meiosis in both male and female germ cells, and re-expressed in oocytes after birth (109, 110). *Pou5f1* expression is required for PGC survival and it has also been suggested to be required for PGC specification (111, 112). The expression of *Nanog* and *Sox2*, is first repressed, but induced again around E7.5 in mouse PGCs with continued expression until initiation of meiosis in female gonads or until mitotic arrest in male gonads (113, 114). *Nanog* expression is not detected in adult testes or ovaries; however, *Sox2* is re-expressed again in primary oocytes. Moreover, *Nanog* and *Sox2* have been shown to be necessary for PGC development and maintenance (115-117).

The expression of POU5F1 and NANOG is detected also in human migratory PGCs and undifferentiated gonocytes or oogonia, however, in contrary to mice, no SOX2 expression was found (70, 118-120). In addition, mouse SSCs in adult testis are expressing POU5F1, but no positive cells were found in adult human testis (121). Other pluripotency genes are also expressed in undifferentiated human germ cells, including LIN28 and SSEA4, whereas the expression of TRA1-60 or TRA1-81 was not observed (118, 119, 122). However, another study reported the expression of TRA1-81 in POU5F1 positive germ cells (123).

The expression of KIT is detected in human germ cells during both fetal and postnatal development, and it is also expressed in hESCs (8). KIT is expressed already in migratory PGCs at 5 weeks of development with continued expression in gonocytes and oogonia, although showing heterogenous expression patterns with other gonocyte/oogonia markers (70, 118, 119, 123, 124). In ovaries, the oocytes in primordial follicles are negative, but start re-expressing KIT at primary follicle stage (73). In adult testis, KIT is expressed in spermatogonia and in the acrosomal region of round spermatids (125).

Although SSEA1 is expressed in mouse embryonic stem cells (mESCs) and not in hESCs, both mouse and human PGCs are positive for SSEA1 (3, 41, 126). SSEA1 is expressed in human migratory PGCs, gonocytes, and oogonia until the onset of meiosis, although some SSEA1 positive cells did not express other germ cell related markers, indicating that it is not a germ cell specific marker (118, 119, 123, 124). In adult human testis, SSEA1 expression was found in few type A spermatogonia (127).

1.3.3 RNA-binding proteins

The germ cell lineage is enriched with RNA-binding proteins (RBPs) that are specifically expressed in the testis and ovary (128). Several RBPs have been identified to contribute to the differentiation and maintenance of germ cell development in diverse model organisms (129).

1.3.3.1 *NANOS* homologs

The *NANOS* gene family is highly conserved and localized to the germ cells among different species (130). *Nanos* was first discovered in *Drosophila*, where it functions in germ cell migration and maintenance by blocking apoptosis and differentiation (131-133). In mice, three *Nanos* homologs exist, from which *Nanos2* and *Nanos3* are important for germ cell development as elimination of the gene results in a complete loss of germ cells in males or both males and females, respectively (134). *Nanos2* is predominantly expressed in male germ cells between E13.5 and E17.5, with continued expression in undifferentiated gonocytes and SSCs in adult testis (135, 136). *NANOS2* functions as a translational repressor for *Stra8*, Synaptonemal complex protein (*Scp*) 3 and Deleted in azoospermia like (*Dazl*), thus, suppressing meiosis (136-138). *Nanos3* is expressed in PGCs as early as E7.25 and until around E14.5 in female germ cells or in male germ cells throughout the fetal period with declining expression after E16.5 (139). *Nanos3* maintains the PGC population during migration via suppression of apoptosis (134, 140). The expression of *Nanos3* is resumed again in postnatal spermatogonia and continues to be expressed in undifferentiated spermatogonia in adults, with a suggested role in the maintenance of SSC population (139, 141).

Similar to mouse, the human *NANOS1* is more ubiquitously expressed, while *NANOS2* and *NANOS3* are expressed specifically in germ cells (142). *NANOS2* is expressed in the fetal testis around 14 weeks of development, with increased expression at 17-20 weeks (86). In the adult testis, *NANOS2* is expressed in the spermatogonia, but also in spermatocytes and some round spermatids (143, 144). In contrary to mouse, *NANOS2* expression is also detected in human fetal ovaries, although at lower levels compared to fetal testis (86, 142, 144).

NANOS3 is expressed in fetal testes and ovaries from around 8 weeks of development, with highest expression around 14–16 weeks (86). The expression of *NANOS3* seems to be largely coupled with *POU5F1* and *PRDM1* expression (123). In the adult testes, *NANOS3* is expressed in spermatogonia, spermatocytes and round spermatids (142, 144). Although *NANOS3* expression is not found in adult mouse ovaries, human oocytes are positive for *NANOS3* in all stages of follicle development (142).

1.3.3.2 *DAZL*

DAZL belongs to the *Deleted in Azoospermia* (*DAZ*) gene family, which consists of two additional members: *DAZ*, a Y-chromosome gene cluster linked to infertility in men; and a highly conserved autosomal gene *BOULE* (145). *DAZ* is present only in humans and old world monkeys, and it is expressed solely in the testis; in spermatogonia and early spermatocytes, while *BOULE* is highly conserved and expressed in later stages of spermatogenesis (145, 146). In contrast, *DAZL* is expressed in both males and females in multiple stages of germ cell development (146).

In mice, *Dazl* expression is initiated during PGC migration and continues to be expressed in both adult testis and ovaries (145, 147-150). Disruption of *Dazl* leads to apoptosis mediated

germ cell loss after PGC migration and defects in progression through meiosis (147, 149, 151, 152). In addition, gonocytes in fetal testis fail to enter mitotic arrest with continued expression of pluripotency markers (153). To elucidate the function of DAZL, its possible mRNA targets have been studied and at least the binding to DEAD (Asp-Glu-Ala-Asp)-box polypeptide 4 (*Ddx4*) and *Scp3* for promoting their translation has been confirmed (154, 155).

In human, DAZL is expressed in the nucleus of germ cells in the 1st trimester, but relocates to the cytoplasm during 2nd trimester, around the time when POU5F1 expression is lost or meiosis is initiated (150, 156). In the adult, DAZL is expressed in spermatogonia, spermatocytes and post-meiotic cells in the testis, and in oocytes in the ovary (145, 150, 157). *DAZL* transcripts are also found in the ICM of blastocysts and in hESCs, although protein expression is not detected in hESCs (158, 159).

1.3.3.3 *DDX4*

The *Drosophila* gene *vasa* has a central role in germ cell specification and in adult gametogenesis, with a null mutation leading to female sterility (160, 161). The mouse *vasa* homolog *Ddx4* (also called *Mvh*) is also specifically expressed in the germ cell lineage (162). *DDX4* is expressed in both male and female gonads from around E11.5 until post-meiotic spermatids and primary oocytes. The disruption of *Ddx4* results in delayed proliferation of PGCs and blocked spermatogenesis in meiosis for males, while females have normal fertility (163). Thus, although *DDX4* is expressed in both male and female germ lines, its exact function is still unclear and may be different between the genders.

In human, *DDX4* expression is found around 7 weeks of development in migratory PGCs, with increased expression after colonizing the gonads and differentiation to oogonia and gonocytes (118, 119, 156, 164). In adult testis, cytoplasmic expression of *DDX4* is detected in spermatogonia and with higher intensity in spermatocytes and spermatids (164). Similarly, oocytes in adult ovaries are positive for *DDX4* with increasing intensity upon follicle development (164).

1.3.4 Meiosis genes

Meiosis is a germ cell-specific cell division, where one round of chromosome replication is followed by two rounds of cell divisions, resulting in daughter cells with half the number of chromosomes. The first meiotic division separates chromosome homologs to two cells, and the second meiotic division separates sister chromatids to two more cells (165). Both meiotic divisions can be further divided into prophase, metaphase, anaphase and telophase stages. The prophase I is the longest stage of meiosis, when chromosome homologs interact and form crossovers of DNA (165). The progression through prophase I can be visualized with a formation of specific protein structure, called synaptonemal complex (SC) (166).

During the first stage of prophase I, called leptotene, SCP2 and SCP3 proteins attach to each pair of sister chromatids (166). During zygotene, the homologous chromosomes start pairing up in a zipper-like fashion and are held together by SCP1 protein (166). The formed SC is

fully formed along the lengths of the chromosomes at pachytene, and chromosomal crossovers can be detected by foci of MutL homolog 1 (167). During diplotene, the SC degrades and chromosomes remain attached from the crossover regions (167). For proper alignment of chromosomes and formation of crossovers, double strand breaks (DSB) in DNA are necessary. The DSB are protected by phosphorylated H2A histone family, member X, which has also been shown to cover the sex chromosomes that do not fully synapse during meiosis (168).

1.4 GERMLINE STEM CELLS

Germline stem cells (GSCs) are the adult tissue stem cells in the gonads that are committed to produce gametes. The GSCs are most extensively studied in the *Caenorhabditis elegans* gonads, *Drosophila* testes and ovary, and mice testes (169). Male GSCs are called SSCs and undergo continuous self-renewal and differentiation into mature spermatozoa in all species studied (170), whereas female GSCs, called OSCs, are rare across the species (171).

1.4.1 Spermatogonial stem cells

SSCs comprise a rare cell population in the testis that upon transplantation can repopulate the recipient basal membrane of the seminiferous tubules, giving rise to complete spermatogenesis in mice (172). SSC transplantation has been described in various different species, making it a golden standard to characterize the functionality of SSCs (173). Xenotransplantation of SSCs of various species, including human, into the testis of a donor mouse has been shown to support SSC maintenance and proliferation (174, 175). However, meiosis was not supported in the xenotransplantation assay, except for rat SSCs (176), most likely due to evolutionary differences between the species. The SSC transplantation technique offers great promise for studying the SSC characteristics and for treating male infertility.

Expansion of the rare SSC population would be necessary for possible clinical use in the future. Although little is known about the self-renewal of mammalian SSCs, progress has been made to identify important signaling pathways. GDNF has been shown to be important for the maintenance and self-renewal of SSCs in mice (177). Long-term *in vitro* cultures were established using GDNF stimulation for mouse SSCs, which exponentially increased in numbers (178). In addition, bFGF has been thought to induce SSC self-renewal (179, 180). Using the same conditions as for mouse, human SSCs were successfully expanded *in vitro*, and were demonstrated to be functional by xenotransplantation even after prolonged culture periods (181, 182). However, additional studies are still needed to replicate these experiments and further characterization for the *in vitro* expanded SSCs should be performed.

1.4.2 Oogonial stem cells

It has long been accepted that the ovary of most mammalian species contains a fixed number of primordial follicles established before birth that declines with age, with no new oocytes formed in the adult life (183). The view of fixed ovarian reserve was challenged in 2004, when the existence of OSCs was proposed in adult mouse ovaries (184). The assumption was

based mainly on calculated rates of oocyte degeneration, which indicated that the follicle pool must be replenished continuously in adulthood. The authors suggested that the OSCs reside in the ovarian surface epithelium, however, in a subsequent study the authors identified bone marrow and peripheral blood as potential source for the OSCs (185). These findings were widely criticized in the field and considered to be open for alternative interpretations (186, 187). To determine the dynamics of human ovarian reserve, extensive histological studies were performed for calculating the number of non-growing follicles in the ovary from conception to menopause. The resulted Wallace-Kelsey model supported the view on fixed ovarian reserve and provided no evidence of neo-oogenesis in normal human physiological ageing (83).

Recently, new evidence for OSCs was reported. Cells isolated from neonatal and adult mouse ovaries were able to proliferate *in vitro* for several months, and were reported to undergo oogenesis upon transplantation into ovaries of infertile mice, and finally resulted in live offspring (188). Based on these results, another group published similar work done on adult mouse ovaries as well as human ovaries (189). Both of these reports were based on isolating a small population of cells from the ovaries using either magnetic-activated cell sorting or fluorescence-activated cell sorting (FACS) with a DDX4 antibody staining for cell surface expression of the protein. This method was in controversy with the previous knowledge of DDX4 expression, which is widely considered to be expressed in the cell cytoplasm (163, 190). The established *in vitro* cultured mouse and human OSCs were reported to spontaneously differentiate into oocyte-like cells in culture, with even a formation of haploid cells (189). However, no polar body formation was shown, which is indicative of the completion of meiosis II and haploid status of oocytes (89). After injecting the human OSCs into an ovarian cortex piece and xenotransplanting the tissue into mice, the authors reported the formation of primordial follicles that consisted of OSC-derived oocyte and recruited granulosa cells from tissue origin (189). Another group, however, demonstrated through live cell imaging that the *Ddx4*-expressing cells from postnatal mouse ovaries did not contribute to oocytes during de novo folliculogenesis (191). Furthermore, the presence of functional OSCs in the adult ovary is in discrepancy with the fixed ovarian reserve and occurrence of menopause (83). It has been proposed that the exhaustion of oocytes even with the existence of OSCs, might be due to ageing of OSCs or the somatic cells in the ovary (192). A potential explanation is that the putative OSCs are not functional in normal physiology, but once isolated from their original niche and placed in a new environment, they are transformed active.

The existence of OSCs is still unclear, however, the recent reports have raised the central dogma of fixed ovarian reserve in question. If proven true, OSCs could be a potential source for basic science to model germ cell development, thus enabling us to acquire a more comprehensive understanding of this process. Possible clinical applications of OSCs include fertility preservation or treatment for infertility (192).

1.5 GERM CELL DIFFERENTIATION FROM PLURIPOTENT STEM CELLS

Differentiation of germ cells from human PSCs offers a promising strategy to examine several aspects of human germ cell development *in vitro*. First, identifying signaling pathways and growth factors that may influence germ cell development, especially PGC specification and meiotic entry. Second, studying the genetic requirements for germ cell development by over expression or silencing of candidate genes. Third, modeling infertility using patient specific iPSCs.

Reports from several research groups suggest that human PSCs have the potential to differentiate to early pre-meiotic germ cells and in few studies, even to post-meiotic germ cells. Although progress has been made during the recent years, improvements for the efficiency and reproducibility of the differentiation are needed.

1.5.1 Differentiation conditions

1.5.1.1 Growth factors

Human ESCs were shown to have the potential to differentiate towards the germ line in 2004, when expression of germ cell related markers was shown in spontaneous EB differentiation (193). This enabled the testing of different growth factors and signaling pathways that may influence human germ cell development. A central growth factor for mouse PGC specification, BMP4, was found to induce germ cell differentiation from hESCs (194). The role of BMP4 in human germ cell formation was further shown by studies using the BMP inhibitor NOGIN, which reduced the germ cell differentiation (195, 196). Thus, BMP4 is one of the most widely used growth factors for germ cell differentiation.

KIT signaling has also been suggested to play a role in human germ cell development, and hESCs differentiated on feeder cells lacking KITLG had less efficient germ cell formation (195). Influence of WNT3A for germ cell differentiation was also suggested through inhibitor studies, however, these findings remain to be verified (196). Other factors that are commonly used in germ cell differentiation are bFGF and RA, because of their role in SSC proliferation and induction of meiosis (197-201). Recently, the conditions used for mouse SSC culture, including bFGF and GDNF growth factors, were directly utilized for human PSC differentiation (202, 203).

1.5.1.2 Differentiation format

The EB differentiation format with or without supplemented factors is often used, however, a variety of other methods have also been reported. Adherent differentiation on gelatin-coated surfaces is used because the total yield of cells after differentiation is higher relative to EBs, and Matrigel is used mainly for better initial attachment of PSCs (159, 204-206). In addition, feeder layers that are used for the maintenance of PSCs have also been used for differentiation experiments (195, 197, 198, 202, 207). Induction of germ cells has also been attempted in co-cultures by using somatic cells that are usually in contact with *in vivo* germ

cells, such as fetal gonad stromal cells, porcine ovarian fibroblasts and human granulosa cells (124, 200, 201).

Majority of the reported studies use the same condition throughout the differentiation, but few step-wise protocols have been described. The combination of differentiation inducing factors has been changed in a step-wise manner in an attempt to mimic *in vivo* germ cell development from PGC specification and proliferation to initiation of meiosis (196, 203, 207). In addition to the changes in medium composition, differentiation format has also been changed from EB differentiation to adherent cultures, or into calcium alginate encapsulated cultures (196, 203). These complex studies, however, are often lacking detailed analysis of the cell population in each step, and the individual effects of growth factors are not tested.

The reported differentiation protocols vary in formats, growth factors and durations (from a few days to more than 10 weeks), making the comparison between experimental conditions difficult. In addition, different strategies for determining the efficiency of differentiation are used between the studies, thus no conclusions for optimal conditions have been made.

1.5.1.3 Isolation of putative germ cells

Differentiation of PSCs results in a heterogeneous cell population. Therefore, isolation of the putative germ cells would be important for detailed characterization and further differentiation experiments. Isolation of cell populations with enriched expression of germ cell related markers were reported by using SSEA1, chemokine (C-X-C motif) receptor 4, or GDNF family receptor alpha 1 (198, 203, 204). However, these markers are not specific for germ cells. Using a combination of unspecific markers, such as SSEA1 and KIT, or TRA-1-81 and KIT, results in a more homogenous cell population (124, 199). Another combination based on CD9 and CD49f expression and negative expression for CD90 and SSEA4 has also been reported (207).

At the moment, there are no germ cell specific cell surface markers known. Therefore, reporter cell lines for intracellular germ cell markers would be a good alternative for isolating putative germ cell populations. PSC lines with POU5F1-enhanced green fluorescent protein (EGFP) constructs have been used, but because undifferentiated PSCs also express POU5F1, this approach is not ideal (196, 201). A more specific isolation of germ cells has been made by using DDX4-EGFP reporters (159, 205, 206).

1.5.1.4 Meiosis in vitro

Differentiation to early pre-meiotic cells is readily seen from PSCs, determined by gene and protein expression profiles. Recently, several groups have also reported the presence of a small population of meiotic cells and even post-meiotic cells, using various differentiation methods (159, 196, 199, 202-204, 206-208). Meiotic cells were identified by the presence of SC formation in meiotic spread preparations by staining for SCP3 protein (159, 196, 206, 208). The percentage of cells with elongated SCP3 staining varied between 1% and 10%, however, they were evaluated and calculated manually, which may cause large variation

between studies. A total population of 150-200 cells was analyzed in these studies, thus, 1-20 cells were found to have initiated meiosis.

Differentiation to post-meiotic cells was determined by the presence of haploid cells (1N) using flow cytometer based DNA content analysis or DNA fluorescence *in situ* hybridization (FISH) for chromosome counting, and in some studies, by staining for post-meiotic male germ cell markers, such as ACROSIN and PROTAMINE 1 (159, 196, 199, 202-204, 206, 207). Of note, there have been no reports about differentiation to post-meiotic oocyte-like cells. It is important to confirm the differentiation to post-meiotic cells by a combination of methods instead of relying on one, because only a fraction of the cells isolated as 1N by FACS were confirmed to have haploid status by FISH (206). Therefore, the efficiency of differentiation to post-meiotic cells is hard to determine, however, it seems to be extremely low.

1.5.1.5 Transplantation assay

Transplantation to seminiferous tubules and observing colony formation is used for the characterization of SSC status and the assay is considered to be the golden standard in the field (95, 173). Recently, human PSCs were transplanted to the seminiferous tubules of busulfan-treated mice for examining their germ cell differentiation potential *in vivo* (208-210). Although extensive tumor formation in the interstitial space was observed in some cases, PSCs were able to colonize the basement membrane of the seminiferous tubules and differentiate into morphologically and immunohistochemically recognizable germ cells. They showed features resembling PGCs and gonocytes, however, no further differentiation was observed. This may be partly due to the limitations of the xenotransplantation assay to support meiotic progression.

1.5.2 Genetic analysis of human germ cell differentiation

In mice, considerable progress in determining the genes related to the germ cell development has been made through gene disruption studies (211). The function of these genes in human germ cell development is largely unknown and mutations correlating with human infertility are rarely found. Human PSCs provide a useful tool to study the genetic requirements for germ cell development through genetic modifications.

The function of DAZL, DAZ and BOULE in human germ cell development was studied in hESCs by silencing and over expression (159). Silencing of DAZL resulted in reduced expression of DDX4, whereas over expression increased DDX4 expression, thus DAZL was found to function in PGC/gonocyte formation. Furthermore, over expression of DAZL, DAZ and BOULE induced meiosis and formation of haploid cells from hESCs, suggesting their role as meiotic regulators. Similar experiments with DDX4 over expression in human PSCs, promoted the differentiation to early germ cells as well as progression through meiosis (206). In another study, *DDX4* was used in combination with *POU5F1*, *SOX2*, *KLF4* and *c-MYC* to reprogram iPSCs (208). These cells had increased potential to differentiate to early germ cells *in vitro*, and *in vivo* by using the xenotransplantation assay.

Genetic studies of germ cell related genes using human PSCs may also provide information about commitment to other cell lineages. Silencing *NANOS3* in differentiated hESCs resulted in reduced expression of germ cell-related genes, pluripotency markers and also ectodermal markers, while endo- and mesodermal genes were not affected (142). Suggesting that *NANOS3* may be required for proper regulation of marker expression for germ cell development, but may also influence ectodermal differentiation. In contrast, over expression of *DPPA3* in hESCs leads to downregulation of neural markers and upregulation of germ cell and endodermal markers (212). Another study also showed negative correlation between germ cell and neural cell differentiation, modulated by *PRDM1* and *SOX2* (106). Over expression of *PRDM1* in hESCs promoted the differentiation to early germ cells, and suppressed the transcription of *SOX2*, whereas silencing of *PRDM1* impaired germ cell differentiation and upregulated neural genes. Furthermore, over expression of *SOX2* skewed the differentiation from germ cells to the neural lineage.

1.5.3 Patient iPSC lines for modeling infertility

The iPSC-technology is especially intriguing for the germ cell development studies. Derivation of iPSCs from patients with genetically caused infertility could be used as an *in vitro* model for human infertility that is often difficult to study using animals. Furthermore, germ cell differentiation from patient-specific iPSCs could lead to a new form of assisted reproductive technology in the future.

The potential of human iPSCs to differentiate to germ cells was shown to be similar to hESCs in 2009, and has been well established since (124). The first patient-specific iPSC line to model infertility was derived from a Klinefelter syndrome (KS) patient, with 47,XXY karyotype (213). Genome-wide transcriptome analysis identified aberrantly expressed genes in KS iPSCs that were associated with autoimmune diseases, progesterone-mediated oocyte maturation and protein catabolic process, which could also be linked to the clinical features of KS. The derived lines had XCI, however, many of the differentially expressed genes that were up-regulated in KS iPSCs, were located on X chromosome, indicating that some genes might escape the XCI and cause an overdosage effect. Germ cell differentiation potential of the KS iPSCs was found to be similar to controls, when spontaneous or BMP4-induced differentiation was used. No further differentiation was attempted, although the defects in germ cell development might be prominent only at the onset of meiosis.

The derivation of Turner syndrome iPSCs, with 45,X karyotype, was recently reported (210). Using a xenotransplantation assay, Turner syndrome iPSCs were capable of forming germ cell like cells (PGCLCs) *in vivo*, indicating that intact X chromosomes are not required for germ cell formation, but may be required for the maintenance of germ cells in adulthood, because of the observed infertility in Turner syndrome patients. In another study, iPSCs derived from infertile men with deletions in the Y chromosome AZF region, were shown to have defects in germ cell differentiation (209). These patient iPSCs formed fewer and poor-quality PGCLCs both *in vitro* and *in vivo* after xenotransplantation.

1.5.4 Differentiation of naïve human PSCs

PGCLCs differentiated from naïve mouse PSCs showed robust capacity for spermatogenesis after injection into testis, and formation of oocytes after injection into ovaries, resulting in healthy offspring (214, 215). In these groundbreaking studies, the mouse germ cell specification pathway was reconstituted *in vitro*, resulting in efficient PGCLC generation from mouse PSCs that had previously been challenging (216). Naïve mouse PSCs were first cultured in bFGF and Activin A for 48h to acquire competence for germ cell fate, followed by induction to PGC fate in EB cultures with BMP4 (215). A robust formation of PRDM1 positive cells (around 40%), and PRDM1/DPPA3 positive cells (around 7%) was observed on day 4. This differentiation protocol, however, was only efficient for naïve PSCs and not for EpiSCs.

The recent advances in the generation of naïve human PSCs enabled testing of the PGCLC induction protocol on human cells (217). Human PSCs with a NANOS3-mCherry knockin reporter were cultured in four-inhibitor-containing medium for conversion to naïve state. Following the PGCLC induction protocol, the cells differentiated efficiently (around 27 %) into NANOS3 and tissue non-specific alkaline phosphatase double positive PGCLCs. In contrast, using conventional primed hESCs, the PGCLC-induction efficiency remained low (0-5 %). Interestingly, even higher efficiency (around 46 %) was reached by omitting the 2-day preinduction step, which was necessary for mouse PSCs. By a thorough study with PRDM1 knockout and SOX17 knockout NANOS3-mCherry hESCs, the authors concluded that SOX17 is a critical signaling component driving human PGC specification, and functions upstream of PRDM1 and NANOS3. In addition, the competence of human PSCs towards the germ line could be influenced by environmental factors.

1.6 INFERTILITY

Infertility is defined as the inability of a couple to conceive after one year of unprotected intercourse. It is a significant health problem that affects about 10-15% of reproductive age couples worldwide, with both male and female factors contributing to the prevalence of infertility (218). The advances in assisted reproductive technology, especially IVF and intracytoplasmic sperm injection (ICSI), have increased the chances of conception (219, 220). However, infertility treatments for those who have few or no germ cells are still largely ineffective.

1.6.1 Klinefelter syndrome

Azoospermia, defined as complete absence of sperm from the ejaculate, is present in about 1% of all men and in 10–15% of infertile men (221, 222). The most common genetic cause of infertility is KS, occurring in 11% of azoospermic men (223). KS is a sex chromosomal aneuploidy with 80–90% of cases having one additional X chromosome resulting in a 47,XXY karyotype, while the remaining cases having higher number of X chromosomes (e.g. 48,XXXY) or mosaicism (46,XY/47,XXY) (224, 225). KS is characterized by small firm testes, androgen deficiency, azoospermia, gynaecomastia, and tall stature (226). In addition,

varying degree of behavioral and learning impairments have been associated with KS (227). Due to variation in the clinical presentation and relatively discrete symptoms, most of the KS cases are diagnosed at adulthood, when the men are seeking treatment for infertility, while only less than 10% are diagnosed before puberty (225). More than 95% of adults with KS have azoospermia and patients often have a severely impaired fertility even before puberty (228). However, KS patients may have focal spermatogenesis in the testis, enabling biological paternity by testicular sperm extraction and ICSI (229).

Germ cell degeneration in KS patients starts already in the fetal period, progresses slowly during childhood, and accelerates during puberty (230). In adults, the testes have extensive fibrosis and hyalinization of the seminiferous tubules, hyperplasia of interstitium, and presence of immature Sertoli cells (230). It is unclear whether the extra X chromosome primarily affects the degeneration of the germ cells or the somatic cells of the testis. XCI, normally occurring in female cells, has also been shown for KS men (231). However, small part of the X chromosome can escape the XCI, which in KS men could result in overdosage of gene products that may compromise the testicular function. It has also been speculated that the XXY germ cells cannot complete meiosis and thus, focal spermatogenesis is only possible when the extra X chromosome is somehow lost from the germ cells, however, both normal and aneuploid spermatozoa have been reported (231). The underlying mechanisms for the germ cell depletion in KS patients remain unclear.

2 AIMS

The general aim of this thesis was to elucidate the genetic requirements for human germ cell development using PSCs and to study the existence of human OSCs.

The specific aims of the four projects were:

- I. To assess the differentiation potential of human iPSCs towards pre-meiotic and post-meiotic germ cells
- II. To study the function of NANOS3 and DAZL in human germ cell development using hESCs
- III. To optimize a clinically relevant iPSC reprogramming method for the derivation of patient specific iPSC line from KS patients
- IV. To isolate and characterize putative OSCs from adult human ovaries

3 MATERIALS AND METHODS

3.1 ETHICS

3.1.1 Human subjects

Human iPSC lines used in Project I were derived at the Stanford University, CA, USA with a written consent from the donor and with the approval from the Institutional Review Board (No. 10368). In project III, skin tissue was biopsied and iPSC lines were derived with a written consent from the donor and with the approval from the Stockholm Regional Ethics Board (Dnr: 2013/1132-32). hESC lines were used with the approval from the Stockholm Regional Ethics Board (Dnr: 454/02 and Dnr: 2013/666-32). In project IV, human ovarian cortical tissue was obtained with a written consent from the donor and with the approval from the Stockholm Regional Ethics Board (Dnr: 2010/549-31/2).

3.1.2 Animal work

In project II, hESCs were transplanted into busulfan treated mice at the University of Pittsburgh, PA, USA with the approval from the Institutional Animal Care and Use Committee (No. 14043505). In project III, human iPSCs were transplanted into mice with the approval from the Stockholm South Ethics committee (Dnr: 5126-11). In project IV, ovarian cells and tissue were transplanted into mice with the approval from the Regional Ethics committee of the University of Gothenburg (Dnr: 11-2013).

3.2 CELL CULTURE

3.2.1 Human PSC maintenance

3.2.1.1 *Project I*

Human PSCs were cultured on γ -irradiated MEFs using culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM)/F-12 or KnockOut-DMEM supplemented with 20 % KSR, 2 mM L-glutamine, 0.1 mM non-essential amino acids (NEAA), 0.1 mM 2-mercaptoethanol (2me), and 10 ng/ml bFGF. Cells were passaged onto fresh feeders in small clumps using 1 mg/ml Collagenase IV.

For feeder-free culture of PSCs, culture plates were coated with 1:30 dilution of Matrigel in KO-DMEM for 2 h at 37 °C or at least over night at 4°C. The above-mentioned medium was conditioned on MEFs for 24 h and used for culturing of human PSCs on Matrigel. Cells were passaged as above.

3.2.1.2 *Project II*

Human ESCs were cultured on Matrigel-coated plates using mTeSR1 medium. Cells were passaged as single cells using Accutase and plated with 5-10 μ M Y-27632 (ROCKi), which was removed from the cultures the next day.

3.2.1.3 *Project III*

Culture plates were coated with 10-20 µg/ml laminin-521 for 2 h at 37 °C or at least over night at 4°C. Human PSCs were cultured on laminin-521-coated plates using mTeSR1 or NutriStem medium. Cells were passaged as single cell using TrypLE Select.

3.2.2 Human PSC differentiation

3.2.2.1 *Project I*

Human PSCs were plated onto Matrigel coated plates using differentiation medium consisting of KO-DMEM supplemented with 20 % FBS, 2 mM L-glutamine, 0.1 mM NEAA, 0.1 mM 2me, and with or without 50 ng/ml BMP4, -7, and -8b. Cells were differentiated up to 14 days and medium was changed once a week.

3.2.2.2 *Project II*

Human ESCs were differentiated on mouse feeder cells using SSC-medium, following previously published method (202). Soriano ES feeder cell line SNL 76/7 STO cells (SNL) were γ-irradiated and plated with a density of 52,000 cells/cm². Human ESCs were collected as clumps using 1 mg/ml Collagenase IV and plated onto SNL-feeders using mTeSR1 medium. The next day, medium was changed to SSC-medium, consisting of Minimum Essential Medium alpha (α-MEM) supplemented with 0.2 % w/v bovine serum albumin (BSA), 1x Glutamax, 10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 50 U/ml and 50 mg/ml penicillin-streptomycin (penstrep), 50 µM 2me, 5 µg/ml insulin, 10 µg/ml holo-transferrin, 30 nM sodium selenite, 60 µM putrescine, 2.36 µM palmitic acid, 0.21 µM palmitoleic acid, 0.88 µM stearic acid, 1.02 µM oleic acid, 2.71 µM linoleic acid, 0.43 µM linolenic acid, 1 ng/ml bFGF and 20 ng/ml GDNF. The SSC-medium was gassed with 90 % N₂, 5 % CO₂, 5 % O₂ gas mixture for 30s before changed to cells every two days. Cells were differentiated up to 14 days.

3.2.3 Primary fibroblast derivation and culture

In project III, skin tissue was biopsied with a 4 mm punch, and cut into small pieces after removal of the dermis. Tissue pieces were cultured in medium consisting of DMEM supplemented with 10 % FBS, and 50 U/ml and 50 mg/ml penstrep. The expanded fibroblasts were passaged with 0.05 % Trypsin.

3.2.4 Ovarian cell culture

Human and mouse ovarian cells isolated by FACS in project IV, were cultured on γ-irradiated MEFs or on 0.1 % gelatin-coated plates using culture medium consisting of α-MEM supplemented with 10 % FBS, 1 mM sodium pyruvate, 1x Glutamax, 0.1 mM NEAA, 0.1 mM 2me, 1x N2-supplement, 50 U/ml and 50 mg/ml penstrep, 10 ng/ml LIF, 1 ng/ml bFGF, 40 ng/ml GDNF, and 10 ng/ml epidermal growth factor, as previously published (189). Cells were passaged using a short incubation with 0.05 % trypsin.

3.3 GENETIC MANIPULATION

3.3.1 Lentiviral transduction

In project I, lentiviral supernatant was produced in 293FT cells by transfecting the cells by lipofection with plasmid of interest and viral packaging constructs VSVG and $\Delta 8.9$. For generating DDX4-EGFP reporter cells, previously published pLVGV vector was used, which contains 2.5 kilobase (kb) *DDX4* promoter region with fused *EGFP* followed by 1 kb three prime untranslated region, and a neomycin resistance gene (159). For over expression, previously published p2K7_{blas} vector carrying *DAZL*, *DAZ2* or *BOULE* open reading frame (orf) under the elongation factor 1-alpha promoter were used (159).

Undifferentiated PSCs cultured on Matrigel were infected using the lentiviral supernatant and 8 μ g/ml polybrene for 24 h at 37 °C. Antibiotic selection of transduced cells was started 48 h after transduction, either with 200 ng/ml geneticin for 7 days (DDX4-EGFP), or with 2 μ g/ml blasticidin for 3 days (over expression).

3.3.2 PiggyBac plasmid lipofection

PiggyBac transposase expression vector (pCX-IFP2) containing *piggyBac* (also known as *IFP2*) gene driven by the CMV early enhancer/chicken beta actin (CAG) promoter was constructed by subcloning BamHI fragment of pBSII-IFP2-orf into pCX-EGFP digested with EcoRI to replace *EGFP* with *IFP2*.

The *piggyBac* transposon vectors were constructed to contain the orf of the gene of interest driven by CAG promoter and followed by internal ribosome entry site and puromycin resistance gene, located between the 5' and 3' long terminal repeat regions. *NANOS3* and *DAZL* orfs were amplified by PCR using cDNA synthesized from human testis RNA as a template. *EGFP* orf was obtained from pEGFP-N1 plasmid. The orfs were cloned into the destination vector using the Gateway LR recombination system.

In projects II and IV, cells were co-transfected with a *piggyBac* transposon and transposase vector in 1:1 ratio using Lipofectamine LTX. Antibiotic selection with 1 μ g/ml puromycin was started 48 h after transfection and continued for 6 days.

3.3.3 Episomal plasmid reprogramming

In project III, episomal plasmids carrying *p53* small hairpin RNA and *POU5F1* (pCXLE-hOCT3/4-shp53), *SOX2* and *KLF4* (pCXLE-hSK), and *L-MYC* and *LIN28* (pCXLE-hUL) were used for reprogramming human fibroblasts into iPSCs, and plasmid carrying *EGFP* (pCXLE-EGFP) was used as a control for transfection (30). Fibroblasts (6×10^5 cells) were electroporated with 1 μ g of each reprogramming plasmid, using 100 μ l tip Neon System with 1650 V, 30 ms, 1 pulse settings. Transfected cells were plated onto non-coated culture plate with fibroblast culture medium for six days. Cells were collected with 0.25 % Trypsin and plated onto laminin-521 or Matrigel-coated plates with a density of 5×10^3 cells/cm². The

following day, medium was changed to NutriStem or mTeSR1. The formed iPSC colonies were manually picked 23–40 days after transfection.

3.4 DNA ANALYSIS

3.4.1 Plasmid copy number

In Project III, genomic DNA was collected from iPSCs using the DNeasy Blood and Tissue kit from Qiagen, and analyzed by quantitative PCR (qPCR) using SYBR Green technology and primers for Epstein-Barr virus nuclear antigen 1 and F-box protein 15 for detecting plasmid and genomic DNA, respectively. Standard curves were prepared from pCXLE-EGFP plasmid and hESC DNA for evaluating plasmid copy numbers per cell. Standard curves for 3–30,000 copies of plasmid and genomic DNA were prepared based on estimated values of 1.096×10^{21} g/bp and 3.0×10^9 bp/haploid human genome.

3.4.2 Karyotyping

In project III, human iPSCs were treated with 10 µg/ml colcemid for 5 h and collected with TrypLE Select. Cells were treated with hypotonic solution (0.4 % potassium chloride) for 40 min and fixed with Carnoy's fixative (1:3 acetic acid:methanol). Metaphase spreads and G-banding analysis was performed at the Genetics Clinic at Skåne University hospital, Sweden, with at least 25 mitoses analyzed per sample.

3.4.3 FISH

Cells isolated by FACS after DNA content analysis in Project I, were attached to slides using cytospin. Cells were fixed with Carnoy's fixative and dehydrated in alcohol series. FISH probe against chromosome 16 was denatured on slides at 85°C for 5 min and hybridized at 37°C over night. Cover slip was applied to slides using mounting medium containing 4',6-Diamidino-2-phenylindole (DAPI) for counterstaining DNA.

3.5 GENE EXPRESSION

3.5.1 qPCR

Total RNA was extracted using the RNeasy Mini kit from Qiagen or PicoPure RNA isolation kit from Life Technologies, with on-column DNase I treatment. Reverse transcription (RT) of RNA to cDNA was done using random hexamers and SuperScript III enzyme. In project I and IV, cDNA was preamplified using Taqman assays as primers in PCR reaction. Gene expression was quantified using Taqman assays and StepOnePlus Real-Time PCR system (Projects II-IV) or Biomark 48.48 dynamic array chip with Biomark machine (Project I). Relative quantity was determined with comparative Ct (threshold cycle) method ($2^{-\Delta\Delta C_t}$).

3.5.2 RNA sequencing

In Project II, total RNA was extracted using the RNeasy Mini Kit with on-column DNase I treatment. cDNA library preparation, sequencing and basic data processing was done at the

National Genomics Infrastructure at SciLifeLab Stockholm, Sweden. cDNA libraries were sequenced with Illumina HiSeq2500. Sequence reads were mapped to human genome using Tophat, and counts (the number of sequences that map to each gene) were calculated using HTseq. Cufflinks was used to calculate the fragments per kilobase per million mapped reads.

In Project IV, single cells were manually picked and cDNA libraries were created as previously described in detail (232). Single-end sequencing was performed on Illumina HiSeq2000 and sequence reads were mapped to the human genome using STAR. The reads per kilobase per million mapped reads were generated using rpkmforgenes software.

3.5.3 RT-PCR

In Project IV, total RNA was isolated using the RNeasy Mini Kit and cDNA was synthesized with iScript cDNA Synthesis Kit. RT-PCR was used with gene specific primers and product was run on agarose gel for semi-quantitative gene expression analysis.

3.6 PROTEIN EXPRESSION

3.6.1 Western blotting

Protein lysates were prepared using radio-immunoprecipitation assay (RIPA) buffer and protease inhibitors. Protein concentration was determined by bicinchoninic acid (BCA) Protein Assay kit with BSA standards using NanoDrop. Samples were denatured using 2me or dithiothreitol and run with sodium dodecyl sulphate-polyacrylamide gel electrophoresis. After running, proteins were transferred using a wet transfer (Project I) or semi-dry (Projects II and IV) transfer method. Transferred blots were blocked using non-fat milk before incubation with primary antibodies. Secondary antibodies conjugated to horseradish peroxidase (HRP) were used. Enhanced chemiluminescence was used for detecting HRP signal using film development or digital imaging.

3.6.2 Immunocytochemistry

Cells were fixed with 4 % formaldehyde, and permeabilized with 0.3 % Triton X-100. Blocking was done with serum from the same species as the secondary antibody. Primary antibodies were incubated over night at 4°C and fluorescence conjugated secondary antibodies were incubated for 1h at room temperature. DAPI was used for counterstaining DNA. For AP staining, Alkaline Phosphatase Detection Kit from Millipore was used (Project III).

3.6.3 Immunohistochemistry

Cross sections of paraffin embedded tissue were deparaffinized in xylene and rehydrated through ethanol series. Antigen retrieval was done in sodium citrate buffer (pH 6) at 96°C, followed by peroxidase and serum blocking. Primary antibodies were incubated over night at 4°C and HRP-conjugated secondary antibodies were incubated for 30 min, followed by

incubation with Tyramide Signal amplification Plus Fluorescein or Cy3 System from Perkin-Elmer. Mounting medium with DAPI was used.

3.6.4 Meiotic spreads

Single cell suspension was incubated with hypoextraction buffer containing 30 mM Tris, 50 mM sucrose, 17 mM citric acid, 5 mM ethylenediaminetetraacetic acid and protease inhibitors. Cell solution was mixed with 100 mM sucrose and either dropped or cytopun onto slides. Samples were fixed with formaldehyde and blocked with serum. Primary antibodies were incubated for 3h and fluorescence conjugated secondary antibodies for 1h. Mounting medium with DAPI was used.

3.7 FACS

3.7.1 DDX4-EGFP expression

In project I, human PSCs transduced with DDX4-EGFP construct were collected after differentiation using Collagenase IV and TrypLE enzymes. Single cell suspension was obtained by filtering cell solution through a 40 µm cell strainer. Non-transduced cells were used as negative control and EGFP-positive cells were sorted based on fluorescence intensity using FACS Aria II.

3.7.2 DNA content analysis

In project I, differentiated human PSCs were collected using Collagenase IV and TrypLE enzymes. Single cell suspension was obtained by filtering cell solution through a 40 µm cell strainer. Cells were fixed with 70 % ethanol for 1h and stained with 0.02 mg/ml propidium iodide in a solution containing 0.1 % Triton X-100 and 0.2 mg/ml RNase A. DNA content was assayed based on staining intensity for propidium iodide on a linear scale using FACS Aria II. Gating for haploid DNA content was set by estimating the distance from the diploid and tetraploid cell peaks and using human semen sample as positive control.

3.7.3 DDX4 expression

In Project IV, human ovarian cortical tissue was prepared for FACS as previously described (233). Briefly, tissue was dissociated into single cells using 400 U/ml Collagenase IV and 1 µg/ml DNase I incubation for 40min in 37°C, with either manual disruption or with GentleMACS tissue dissociator. Mouse tissue was dissociated by mincing and digestion with 800 U/ml Collagenase IV and 1 µg/ml DNase I. Cultured human ovarian cells were dissociated into single cells using 10 min 1mg/ml Collagenase IV and 2 min TrypLE incubations. Cells were blocked with serum from the same species as the secondary antibody, incubated with DDX4 antibody for 20 min and with fluorescence conjugated secondary antibody for 20 min. 7-Aminoactinomycin D was used for dead cell discrimination. Secondary antibody only, and isotype samples were used as negative controls and cells were sorted based on fluorescence intensity using FACS Aria II (mouse cells) or FACS Aria III (human cells).

3.8 TRANSPLANTATION

3.8.1 Xenotransplantation into seminiferous tubules

In Project II, immunodeficient mice (NCr nu/nu) were treated with 40 mg/kg of busulfan to deplete germ cells. After five weeks or more, 1×10^6 hESCs were injected into the seminiferous tubules via cannulation of the efferent ducts. After eight weeks, the testes were collected for whole mount staining or for paraffin embedding and cross sectioning.

For whole mount staining, the seminiferous tubules were dispersed using 1 mg/ml Collagenase IV and DNase I, and fixed with 4 % formaldehyde. Primary antibody against primate testis cells and fluorescence conjugated secondary antibody were used for detecting human cells. For cross sectioning, testes were fixed with 4 % formaldehyde, dehydrated and embedded into paraffin. Serial cross sectioning and hematoxylin and eosin (HE) staining was done at the Department of Laboratory Medicine at Karolinska Institutet, Stockholm, Sweden.

3.8.2 Teratoma assay

In Project III, human iPSCs were detached as small cell clumps by manual scraping, and plated into ultra-low adhesion plates with mTeSR1 or NutriStem medium with 10 μ M Y-27632 for 24 h. Cell suspension (approximately 1×10^6 cells) was mixed with undiluted Matrigel and injected subcutaneously into SCID/Beige mice. Mice were sacrificed and tumors collected at 3–8 weeks after injection. Tumors were fixed in 4 % formaldehyde, dehydrated, embedded in paraffin and cross-sectioned for HE staining at the Department of Laboratory Medicine at Karolinska Institutet, Stockholm, Sweden.

3.8.3 Ovarian cell transplantation

3.8.3.1 Human cells

Human ovarian cells labeled with EGFP (approximately 3,000 cells), were injected into human ovarian cortical tissue piece (2 x 2 x 1 mm). The tissue piece was xenografted under the kidney capsule of recipient SCID female mouse. Xenografts were collected 1 week, 2 weeks or 4 weeks after transplantation, fixed in 4 % formaldehyde, dehydrated, and embedded in paraffin for cross-sectioning. Fluorescent images were taken before HE staining.

3.8.3.2 Mouse cells

Red fluorescent mouse ovarian cells from *mTmG* (membrane-targeted Tomato and Green) females were injected into ovaries of adult female mice treated sterile with busulfan (30 mg/kg) and cyclophosphamide (120 mg/kg). Approximately 10,000 cells were injected into each three locations per ovary. The ovaries were collected 1 week, 2 weeks or 6 weeks after injection, fixed in 4 % formaldehyde, dehydrated, and embedded in paraffin for cross-sectioning. Fluorescent images were taken before HE staining.

3.9 STATISTICS

Statistical significance between two groups was tested using unpaired t-test. For comparing multiple groups, one-way Analysis of variance (ANOVA) or two-way ANOVA with correction for multiple comparisons were used. Testing was done with Prism6 software and significance was accepted at $p < 0.05$.

Differential gene expression analysis for mRNA sequencing data was done using edgeR analysis and R software. Significance was accepted at false discovery rate < 0.05 .

4 RESULTS AND DISCUSSION

4.1 GERM CELL DIFFERENTIATION POTENTIAL OF IPSCS (PROJECT I)

HESCs can differentiate to pre-meiotic germ cells in response to BMP stimulation, and to meiotic and post-meiotic germ cells by over expressing the intrinsic meiotic regulators, *DAZL*, *BOULE* and *DAZ* (159). In Project I, we followed the same experimental approach to evaluate the germ cell differentiation potential of human iPSCs reprogrammed from fetal fibroblasts: iPS(IMR90), and from adult fibroblasts: iHUF4, using two hESC lines as controls: H9 and HSF1.

4.1.1 Pre-meiotic germ cells

After 7 and 14 days of BMP4, -7 and -8b stimulation, hESCs and iPSCs had induced protein expression of germ cell markers DDX4 and DAZL, in similar levels between the cell lines. Interestingly, low expression of DDX4 or DAZL was also detected in undifferentiated iPSCs, indicating the presence of a subpopulation of cells expressing germ cell markers even in the undifferentiated culture conditions.

Gene expression of germ cell related markers was variable between the cell lines. Interferon induced transmembrane protein 1 (*IFITM1*) expression was higher in iPSCs relative to hESCs at day 7, and in iHUF4 relative to the other cell lines at day 14 of differentiation, whereas *PRDM1* expression was higher for HSF1 relative to H9 and iPS(IMR90) cell lines at day 7 of differentiation. Expression of *PELOTA* was similar between the cell lines after differentiation; however, higher expression was observed for undifferentiated iPSCs relative to hESCs. In addition, *IFITM1* expression was higher for undifferentiated HSF1 and iPS(IMR90) cells relative to H9 and iHUF4 cells, and *PRDM1* expression was lower in iHUF4 cells relative to the other undifferentiated cell lines.

Cells were transduced with DDX4-EGFP construct and analyzed by flow cytometer after 7 days of differentiation. Approximately 2 % DDX4-EGFP positive cells were observed for hESC lines and 5 % for iPSC lines. The DDX4-EGFP positive and negative cells were isolated by FACS, and DDX4 expression in positive cells was confirmed by immunocytochemistry. Gene expression analysis revealed that several germ cell related markers were enriched in the DDX4-EGFP positive population: DNA meiotic recombinase I, Nuclear receptor subfamily 6, group A, member 1, *IFITM1*, *PRDM1*, *DPPA3*, *PELOTA* and *POU5F1*, whereas markers for the other germ layers were only detected in the negative population: GATA binding protein 6, Actin alpha cardiac muscle 1, Neural cell adhesion molecule 1, and Keratin 7, type II.

4.1.2 Meiotic germ cells

Cells were transduced with over expression constructs for *DAZL*, *BOULE* and *DAZ*, and differentiated up to 14 days. Meiotic initiation was observed in all cell lines in a subset of cells by immunocytochemistry for SCP3 in meiotic spread preparations, with punctate

staining pattern indicating early leptotene stage and elongated staining pattern indicating zygotene, pachytene or diplotene stages of meiotic prophase I. Similar percentage of cells with punctate staining was observed for H9, HSF1 and iPS(IMR90) cells (9.36 %, 7.88 %, and 7.13 %, respectively), whereas a higher percentage for iHUF4 cells was observed (20.86 %). The percentage of cells with elongated staining was similar between H9 and iHUF4 (0.75 % and 1.23 %), and HSF1 and iPS(IMR90) (4.13 % and 4.63 %). No elongated staining was found for differentiated cells without over expression, but surprisingly, a rare cell with elongated staining was observed in undifferentiated iPS(IMR90) and iHUF4 cultures.

4.1.3 Post-meiotic germ cells

The completion of meiosis and formation of haploid cells was assessed by DNA content analysis using FACS, followed by DNA FISH. Human semen sample was used to set FACS gating to correspond the 1N cell population, and a small percentage of cells (> 2 %) was isolated from differentiated hESCs and iPSCs. The haploidy of the sorted cells was tested by DNA FISH for chromosome 16; after over expression, 13–21 % of the cells had a single copy of chromosome 16, whereas no haploid cells were confirmed for cells differentiated without over expression.

Perinuclear ACROSIN expression, characteristic for spermatids, was observed for 35–72 % of 1N sorted cells. The higher percentage of ACROSIN staining relative to haploid cells suggests that ACROSIN expression may precede completion of meiosis. This was further supported by the observed ACROSIN positive cells and lack of haploid cells in samples without over expression. Furthermore, the percentage of ACROSIN positive cells was higher in over expressed sample (72 %) relative to sample without over expression (31 %).

4.1.4 Discussion

The differentiation of human fetal somatic cell-derived iPSCs to early stage germ cells had been previously shown (124), however, the potential of adult somatic cell-derived iPSCs to generate germ cells had not been reported, nor had the ability of iPSCs (from fetal or adult sources) to differentiate to meiotic and post-meiotic germ cells. We showed that human fetal and adult somatic cell-derived iPSCs could differentiate to pre-meiotic germ cells in response to BMP-signaling, in a similar manner to hESCs. In addition, the over expression of *DAZ* family proteins in iPSCs, like in hESCs, induced the initiation of meiosis, and furthermore, the formation of haploid cells with characteristic staining of ACROSIN for spermatids. With these results (summarized in Figure 5), we show that iPSCs provide a useful platform to study human germ cell development.

We observed some differences between iPSCs and hESCs: the expression of germ cell markers and elongated SCP3 staining in undifferentiated iPSCs, and a higher percentage of DDX4-EGFP cells from iPSCs relative to hESCs upon differentiation. Thus, our data suggest that germ cell differentiation may occur more spontaneously in iPSCs than in hESCs. This may be linked to induced expression of pluripotency markers, the process of reprogramming, or a preferential differentiation to germline rather than somatic cell lineages. However,

further studies with additional cell lines are needed to elucidate the differences between hESCs and iPSCs and the possible mechanisms behind them.

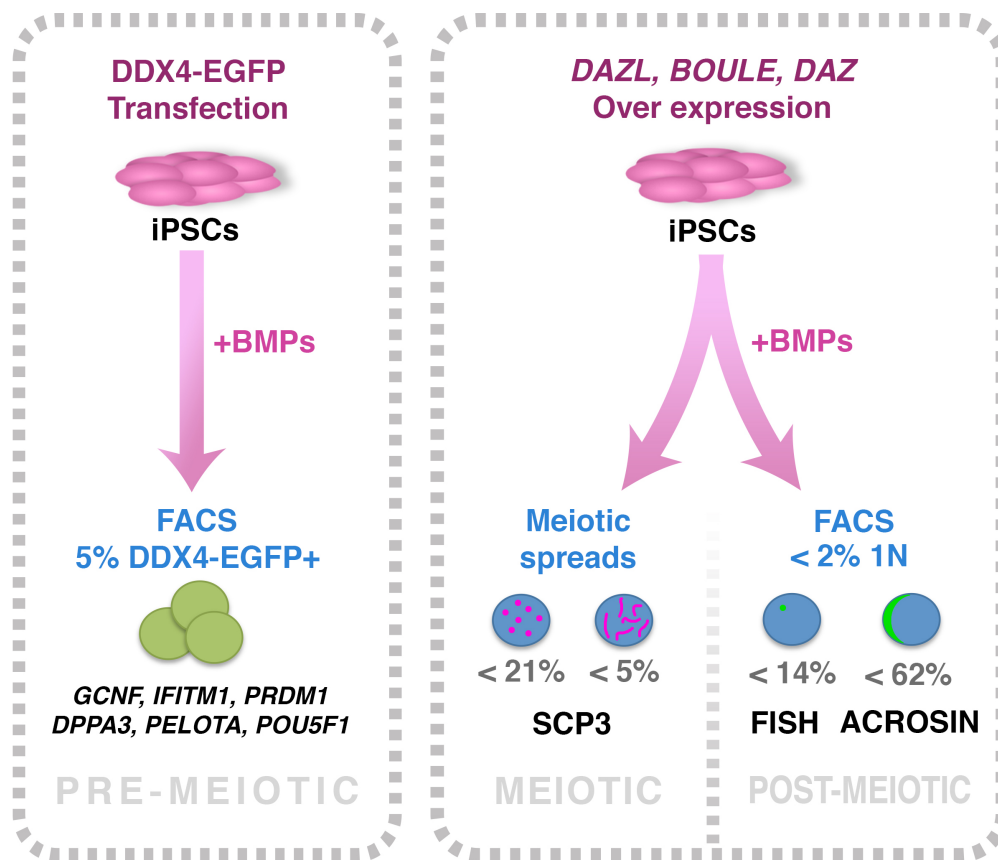


Figure 5. Human induced pluripotent stem cells (iPSCs) formed pre-meiotic, meiotic and post-meiotic germ cells upon differentiation with bone morphogenetic proteins (BMPs). Human iPSCs transfected with DDX4-EGFP reporter construct formed 5 % positive cells, which expressed several pre-meiotic germ cell genes. Upon over expression of DAZL, BOULE and DAZ, human iPSCs entered into meiosis shown by punctate (< 21 %) or elongated (< 5 %) SCP3 staining pattern. In addition, a small percentage (< 2 %) of haploid (1N) cells were isolated by fluorescence-activated cell sorting (FACS), from which < 14 % were confirmed to be haploid by fluorescent *in situ* hybridization (FISH), and < 62 % had a perinuclear ACROSIN staining.

4.2 FUNCTION OF NANOS3 AND DAZL (PROJECT II)

The germ cell specific RBPs, NANOS3 and DAZL, are important for germ cell development in mice, however, their function in human is poorly understood. In Project II, we used hESCs as a tool to study the effects of NANOS3 and DAZL via over expression. We analyzed the cells cultured in pluripotency maintaining conditions, and after *in vitro* and *in vivo* germ cell differentiation.

4.2.1 Pluripotency maintaining conditions

Stable transfection of hESCs with *piggyBac* mediated genomic integration of over expression constructs was confirmed by qPCR, immunocytochemistry and Western blotting. Cells could be cultured in pluripotency maintaining conditions for several passages, while maintaining the over expression of NANOS3 or DAZL. Majority of the *NANOS3* transfected cells (*pbNANOS3*) expressed NANOS3 protein and grew in colonies with similar morphology to

undifferentiated hESCs. For *DAZL* transfected cells (*pbDAZL*), we observed cells outside the colonies that stained positive for *DAZL*, while majority of the cells in the colonies seemed negative.

The expression of *POU5F1* and *NANOG* was similar in *pbNANOS3* and *pbDAZL* cells relative to control cells transfected with an empty vector (*pbMOCK*). By immunocytochemistry, similar staining pattern of *POU5F1* and *NANOG* was observed for *pbNANOS3* and *pbDAZL* cells relative to *pbMOCK* cells, although *pbDAZL* cells outside the colonies were negative.

Global transcriptional analysis by mRNA sequencing revealed very similar gene expression profiles of *pbNANOS3* and *pbDAZL* cells relative to *pbMOCK*; four genes were up regulated in *pbNANOS3* cells, and eighteen genes were up regulated and eight down regulated in *pbDAZL* cells. Among the up regulated genes in *pbNANOS3* cells, Olfactomedin 2 (*OLFM2*) has been shown to mediate TGF β response with possible role in cell migration (234, 235), and Protein kinase C substrate 80K-H (*PRKCSH*) has a role in inhibition of apoptosis and induction of cell proliferation (236).

Among the up regulated genes in *pbDAZL* cells, *IFITM3* and *BMP7* are associated with pre-meiotic germ cells (101, 237), and Huntingtin interacting protein 1 related (*HIP1R*), Inositol-3-phosphate synthase 1 (*ISYNA*), and Jagged 2 (*JAG2*) are associated with meiotic or post-meiotic germ cells (238-240). In addition, two of the up regulated genes have a role in cell cycle arrest: p53-induced death domain protein (*PIDD1*) and Reprimo (*RPRM*) (241, 242). Four out of the eight down regulated genes in *pbDAZL* cells have been shown to play a role in cell migration: Chemokine (C-F-C motif) ligand 5 (*CXCL5*), Gamma-aminobutyric acid A receptor, pi (*GABRP*), Family with sequence similarity 110, member C (*FAM110C*), and Lymphocyte cytosolic protein 1 (*LCPI*) (243-246).

4.2.2 *In vitro* germ cell differentiation

We followed the previously published method for *in vitro* germ cell differentiation using SSC-medium and SNL-feeders up to 14 days (202). We found the induction of germ cell-related Promyelotic leukemia zinc finger ortholog (*PLZF*) and *PRDM1*, and down regulation of *POU5F1* and *NANOG* in *pbNANOS3*, *pbDAZL*, and *pbMOCK* cells upon differentiation. In addition, *NANOS3* expression was induced in *pbDAZL* and *pbMOCK* cells, whereas *DAZL* expression was down regulated in *pbNANOS3* and *pbMOCK* cells upon differentiation. Notably, we also observed the induction of Paired box 6 (*PAX6*) and maintained expression of *SOX2* in all cell lines, suggesting a heterogeneous differentiation towards the neural lineage, in addition to germ cells.

Interestingly, we found that *DAZL* expression was significantly lower in undifferentiated *pbNANOS3* cells, but higher at day 14 of differentiation, relative to *pbMOCK*. We also observed a lower *PLZF* and *PAX6* expression in *pbNANOS3* cells relative to *pbMOCK* cells at day 7 of differentiation, suggesting a delayed differentiation of *pbNANOS3* cells. Furthermore, *POU5F1* and *NANOG* expression persisted longer for *pbNANOS3* and *pbDAZL*

cells relative to *pbMOCK* cells, suggesting either differentiation to PGCLCs or remaining undifferentiated cells.

4.2.3 *In vivo* germ cell differentiation

We tested the *in vivo* germ cell differentiation potential of *pbNANOS3* and *pbDAZL* cells via xenotransplantation into the seminiferous tubules of busulfan treated mice, as previously described (208, 209). The xenografts were collected eight weeks after transplantation and human cells were identified in whole-mount staining by an antibody against primate testis cells, or in paraffin embedded cross sections by a human cell specific antibody against Nuclear mitotic apparatus protein 1. By whole-mount staining of *pbDAZL*-transplanted testis, we found six human cell-derived cell colonies, with characteristic features of spermatogonial cell colonies: ovoid shape cells with high nuclear to cytoplasmic ratio, connected by intercytoplasmic bridges. For *pbNANOS3*-transplanted testis, a clump of human cells lacking the spermatogonial features was observed, whereas no positive cells were found in the *pbMOCK*-transplanted testes.

Majority of the xenografts had a dominating tumor component and at least one testis transplanted with *pbNANOS3*, *pbDAZL*, or *pbMOCK* cells had teratoma formation. The seminiferous tubules of the xenografts had a similar percentage of human cell colonization (2.4-8.7 %), and most of the colonized tubules were full of human cells. We did not, however, observe any human cells positive for DDX4, indicating that the naïve niche of the tubules may have been disrupted and therefore could not support the germ cell differentiation to DDX4 positive cells.

4.2.4 Discussion

We utilized hESCs as a platform to study the function of NANOS3 and DAZL via over expression. For NANOS3, we found candidate genes that could mediate the inhibition of apoptosis and induction of migration in germ cells that are known functions of *Nanos3* in mice (134, 140). In addition, we found a delayed differentiation and prolonged expression of pluripotency markers in *pbNANOS3* cells, suggesting a possible role for NANOS3 in suppression of differentiation, similar to Nanos in *Drosophila* (132, 133). For DAZL, we identified several candidate genes that may mediate its known functions in germ cell differentiation and cell cycle arrest (149, 152, 153). Furthermore, we found that *pbDAZL* cells formed spermatogonial cell-like colonies after xenotransplantation, supporting the role of DAZL in germ cell differentiation. In addition, we found a possible new function for DAZL in inhibition of cell migration.

Based on these results, we propose a model summarized in Figure 6, for the NANOS3 and DAZL mediated functions in human germ cell development. We suggest that NANOS3 and DAZL have largely opposing functions in germ cell development, with NANOS3 inducing cell migration and suppressing differentiation, while DAZL suppresses cell migration but induces differentiation. In addition, our data suggest a possible regulation of *DAZL*

expression by NANOS3, however; further studies are needed to confirm the interaction and possible direct binding of NANOS3 to *DAZL* mRNA.

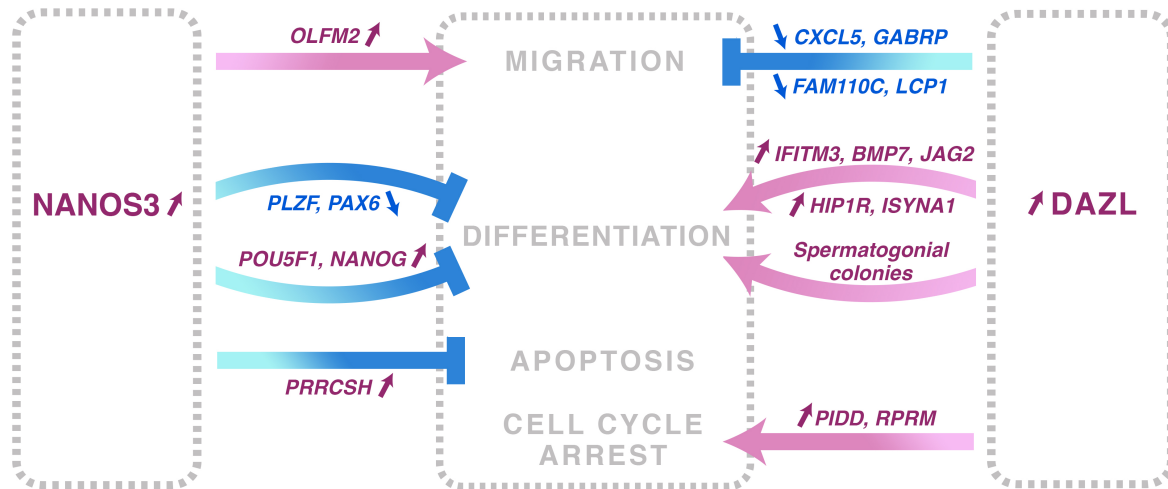


Figure 6. Proposed model for NANOS3 and DAZL mediated functions in germ cell development. NANOS3 may function in inhibition of apoptosis, suppression of differentiation, and promoting cell migration, while DAZL may block cell migration and induce germ cell differentiation and cell cycle arrest.

4.3 INTEGRATION-FREE IPSCS FROM KS PATIENT (PROJECT III)

Improvements for iPSC reprogramming conditions are needed in order to establish safe, chemically defined, and xeno-free cells for translational and possible clinical applications. In Project III, we used non-integrating episomal plasmids for iPSC reprogramming, combined with chemically defined and xeno-free iPSC culture conditions. Furthermore, using the developed conditions, we reprogrammed patient-specific iPSCs from fibroblasts derived from KS patients to establish an *in vitro* model to study the effects of supernumerary X chromosome.

4.3.1 Reprogramming on laminin-521

We derived human dermal fibroblasts (HDFs) from skin tissue biopsied from healthy adult males, and tested the reprogramming efficiency in feeder-free conditions using episomal plasmids: pCXLE-hOCT3/4-shp53, pCXLE-hSK and pCXLE-hUL (30). HDFs were transfected with the episomal plasmids by electroporation and cultured in fibroblast culture medium for 6 days, before reseeding the cells onto laminin-521 or Matrigel coated plates. The following day, culture medium was changed to chemically defined and xeno-free NutriStem medium, and cells were transferred to low oxygen conditions, which has been shown to increase iPSC reprogramming efficiency and promote the derivation of female hESCs with active X chromosomes (247, 248). We observed several small cell colonies that were positive for AP, on both laminin-521 and Matrigel coated plates by 15 days after transfection. However, significantly higher reprogramming efficiency was calculated for laminin-521 relative to Matrigel coated plates: 0.52 % vs. 0.36 %, respectively ($p < 0.05$).

For further characterization of iPSCs, we picked single colonies from laminin-521 and expanded them further. The iPSCs grew as uniform monolayer with typical hESC-like morphology on laminin-521 (5). The loss of episomal plasmids was confirmed by qPCR analysis for genomic DNA and a normal karyotype was confirmed by G-banding. The pluripotency was confirmed by the expression of pluripotency genes and the formation of teratoma after injection into SCID mice.

4.3.2 KS iPSCs

We collected skin tissue from azoospermic men diagnosed with KS (47,XXY), and derived iPSCs by using the optimized laminin-521 reprogramming conditions. With a special focus on the XCI status, we analyzed the gene expression level of *XIST*. We found that the KS fibroblasts and KS iPSCs had a similar or higher level of *XIST* expression relative to female fibroblasts, indicating XCI. To confirm the XCI in KS iPSCs, we analyzed the expression of Histone H3 trimethylated Lysine 27, a repressive chromatin marker, by immunocytochemistry. We observed an accumulated positive signal in the nuclei of KS fibroblasts and KS iPSCs, further indicating an inactive X chromosome.

4.3.3 Discussion

We achieved an efficient reprogramming of integration-free iPSCs in combination with chemically defined and xeno-free laminin-521 and NutriStem medium, summarized in Figure 7. The reprogramming efficiency using episomal plasmids was significantly higher on laminin-521 (0.52 %) relative to Matrigel (0.36 %) and higher than in the original report using mouse feeder cells (0.39 %) (30). Furthermore, the recently reported reprogramming efficiency with the same episomal plasmids but using laminin-511 E8 fragments was extremely low (0.077 %) (39). Thus, laminin-521 supports an efficient reprogramming of iPSCs. Indeed, another recent report described the use of laminin-521 in iPSC reprogramming, with an efficiency of 0.3 %; however, an excisable polycistronic lentiviral method was used, which requires an additional manipulation step to excise the vector for the derivation of transgene-free iPSCs (249). Our new reprogramming conditions provide critical improvements towards deriving clinically safe iPSCs in an efficient manner. However, further optimization for the primary fibroblast derivation and culture conditions are still needed in order to produce iPSCs with a completely xeno-free reprogramming process.

We derived patient-specific iPSCs from fibroblasts of donors with KS. We showed that the derived KS iPSCs had XCI, suggesting that low oxygen condition may not promote X chromosome reactivation during reprogramming or that XCI may be a characteristic feature of XXY cells. In support of the latter hypothesis, one earlier report on KS iPSCs also described XCI for the cells (213), however, further studies are required to determine whether or not it's possible to derive KS iPSCs with both X chromosomes active. Regardless, the KS iPSCs provide a useful model to study sex chromosome aneuploidy and the possible defects in germ cell differentiation.

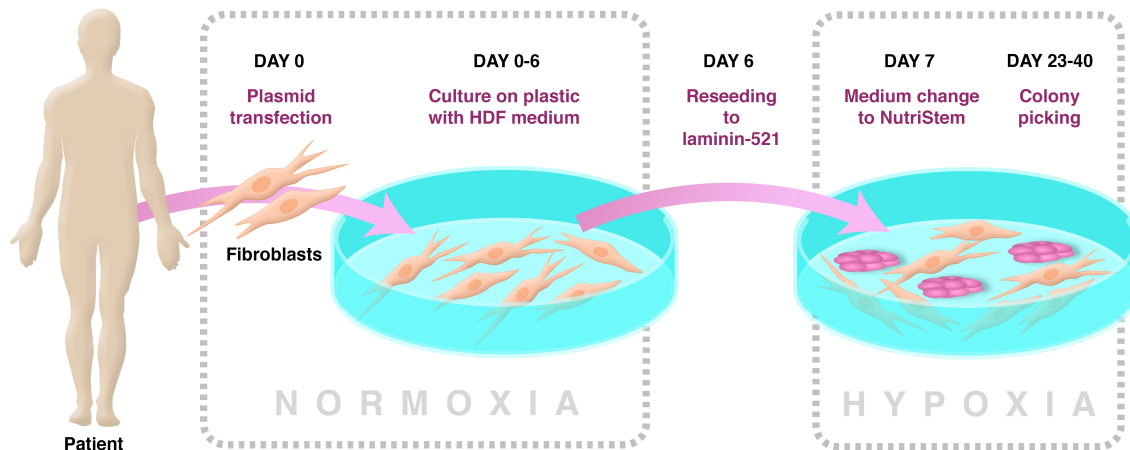


Figure 7. Reprogramming of patient fibroblasts in clinically relevant culture conditions. Primary fibroblasts are transfected with episomal reprogramming plasmids and cultured on plastic with human dermal fibroblast (HDF) medium in normoxia for 6 days, until they are reseeded onto laminin-521 coated plates. On day 7, culture medium is changed to chemically defined and xeno-free NutriStem medium and cells are cultured in hypoxia until reprogrammed cell colonies are picked around day 23-40 for cell line expansion.

4.4 CHARACTERIZATION OF PUTATIVE OSCS (PROJECT IV)

A recent report has raised the central dogma of fixed ovarian reserve in question with the isolation of mitotically active and functional OSCs from adult human ovaries (189). In Project IV, we followed the reported protocol for the isolation of putative OSCs from human and mouse ovaries for further characterization by gene and protein expression analysis, and by transplantation assay.

4.4.1 Isolation of cells with DDX4 antibody-based FACS

In germ cells, DDX4 is expressed in the cytoplasm of the cell and therefore the use of DDX4 as a cell surface marker for FACS is controversial. Regardless, similar to the previous report, we were able to isolate a small population of cells ($12.5\% \pm 4.03\%$) from human ovarian cell preparation that appeared positive for cell surface expression of DDX4 relative to isotype and secondary antibody controls. However, we did not detect any *DDX4* mRNA expression in the isolated “DDX4-positive” cells by qPCR or by single-cell mRNA sequencing, instead, we found that genes expressed in differentiated ovarian somatic cells were frequently detected. In addition, we did not detect any *Ddx4* mRNA expression in the isolated “DDX4-positive” cells from mouse ovarian cell preparation, but we did detect the expression of *Foxl2*, a marker of granulosa cells, at similar levels to that of the ovary. When we tested the DDX4 antibody-based FACS method with mouse cells from organs that do not express DDX4 (liver, spleen, and kidney), we found that similar “DDX4-positive” cell population could be isolated. These results showed that the use of the DDX4 antibody in the FACS method does not select for DDX4-expressing cells.

4.4.2 Functional assay for putative OSCs

We established *in vitro* cultures of the isolated “DDX4-positive” and “DDX4-negative” human ovarian cells as previously described (189). The cultured cells did not express DDX4 by immunocytochemistry, however, we found that both the “DDX4-positive” and “DDX4-negative” cells appeared positive using the DDX4 antibody-based FACS, further confirming the unspecificity of the reported FACS method. Although the “DDX4-positive” ovarian cells did not express DDX4, we tested whether they could still be functional OSCs and generate oocytes after transplantation, as previously described (189).

We labeled the cultured “DDX4-positive” human ovarian cells with stable EGFP expression and injected them into human ovarian cortical tissue pieces, which were subsequently xenografted into SCID mice (Figure 8). We analyzed the grafts at 1, 2 or 4 weeks after transplantation and found EGFP-positive cells in the vicinity of the injection sites. However, we did not observe any EGFP-positive oocytes, indicating that the isolated “DDX4-positive” cells are not functional stem cells that can generate oocytes.

The “DDX4-positive” cells isolated from ovaries of *mTmG* mice, with all cells carrying red fluorescence, were expanded in culture for functional testing. The cells were injected into mouse ovaries and analyzed at 1, 2 or 6 weeks after injection. Similar to the results with human ovarian cells, we did not observe any newly formed oocytes from the injected red fluorescent “DDX4-positive” cells.

4.4.3 Discussion

We showed that the use of DDX4 antibody in FACS, which is a key tool in purifying the reported OSCs, does not select for DDX4-expressing cells. Furthermore, we showed that the human and mouse ovarian cells isolated with this method, did not form oocytes after transplantation. Thus, the isolated cells are neither specific DDX4-expressing cells nor are they functional OSCs. In contrast to the previous report (189), we found no evidence for the existence of OSCs in adult human or mouse ovaries.

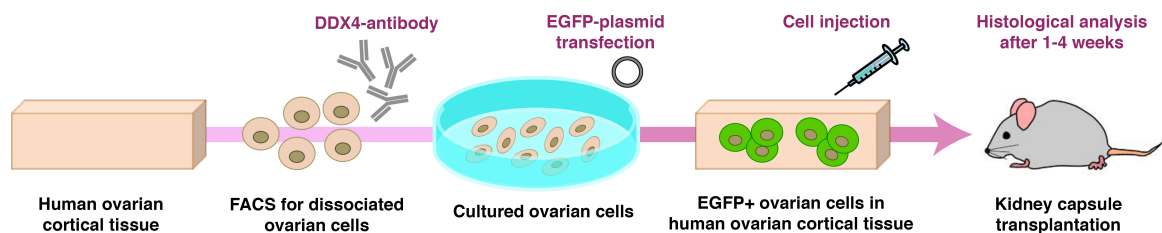


Figure 8. Functional testing for oogonial stem cells (OSCs). Human ovarian cortical tissue was dissociated to single cells and putative OSCs were isolated by DDX4 antibody-based FACS for *in vitro* culturing. The cultured ovarian cells were labeled green with EGFP-plasmid transfection and injected into human ovarian cortical tissue, which was transplanted under the kidney capsule of a mouse. After 1–4 weeks, the transplanted tissue was collected for histological analysis, to assess whether or not green fluorescent oocytes were formed from the injected cells.

5 CONCLUSIONS

In this thesis, we evaluated the germ cell differentiation potential of human iPSCs and used hESCs as a tool to study the function of germ cell specific RBPs: NANOS3 and DAZL. In addition, we optimized iPSC reprogramming method towards xeno-free and chemically defined conditions, and reprogrammed patient-specific iPSCs from infertile patients diagnosed with KS. Furthermore, we assessed the existence of OSCs in adult human ovaries. The main findings are listed below.

- Human fetal- and adult somatic cell –derived iPSCs could differentiate to pre-meiotic germ cells *in vitro*, with similar or even higher efficiency than hESCs. In addition, by over expressing intrinsic meiotic regulators: DAZL, BOULE and DAZ, human iPSCs formed meiotic and post-meiotic haploid germ cells *in vitro*, in a similar manner to hESCs. These data show that human iPSCs, in addition to hESCs, offer a valuable tool to study human germ cell development in all differentiation stages.
- Over expression of NANOS3 in hESCs induced the expression of genes that are associated with mediating TGF β –signaling and cell migration, and inhibition of apoptosis. Furthermore, NANOS3 over expression delayed the *in vitro* differentiation of hESCs by maintaining the expression of pluripotency genes and suppressing the expression of differentiation genes. Thus, human NANOS3 protein may function in PGC migration, inhibition of apoptosis, and suppressing of differentiation that are all conserved functions of NANOS family proteins in different model organisms.
- Over expression of DAZL in hESCs induced the expression of genes related to cell cycle arrest and germ cell differentiation, while several genes related to cell migration were down regulated. In addition, DAZL over expressed hESCs formed spermatogonial-like cell colonies after transplantation into the seminiferous tubules of mice. These data indicate that the known function of DAZL in germ cell differentiation and cell cycle arrest is conserved also in human. Furthermore, DAZL may also have a role in a novel function in inhibition of cell migration.
- Laminin-521 and NutriStem medium supported an efficient reprogramming of iPSCs using integration-free episomal plasmids, providing critical improvements towards establishing clinically safe iPSCs. In addition, iPSCs from infertile KS patients were derived for future studies of X chromosome aneuploidy and defects in germ cell differentiation.
- The DDX4 antibody –based FACS method for isolation of putative OSCs did not select DDX4-expressing cells. In addition, the isolated cells from adult human or mouse ovaries did not form oocytes after transplantation, indicating the cells are not functional OSCs. Thus, no supporting evidence for the existence of OSCs in adult human or mouse ovaries was found

6 FUTURE PERSPECTIVES

Human germ cell specification and differentiation is poorly understood, which is largely due to the scarcity of PGCs and the inaccessibility of the early human embryos. Human PSCs offer a promising tool to model germ cell differentiation *in vitro* and to identify intrinsic genes regulating the germ cell development. We showed that both hESCs and iPSCs have the potential to differentiate to germ cells; however, the efficiency of differentiation is still low and results in a heterogeneous cell population. Fluorescence reporter constructs for germ cell –specific expression can be used to enrich for the differentiated germ cells and to isolate them for further experiments. In addition, the constructs could be used for identifying cell surface markers for the isolation of germ cells without genetic manipulation and for screening growth factors and small chemical libraries for more efficient germ cell differentiation.

The initiation and progression through meiosis, a hallmark of germ cell development, is still a challenge for the *in vitro* differentiation of PSCs. Over expression of *DAZL*, *BOULE* and *DAZ* has been shown to induce meiosis in hESCs (159), and we confirmed the same effect for iPSCs. However, extremely low percentage of cells initiated or completed meiosis even with the over expression, which may be partly due to translational regulation of the *DAZ* family genes. We observed that hESCs transfected with a *piggyBac* over expression construct for *DAZL* had high expression of *DAZL* mRNA, while only a subpopulation of the cells expressed DAZL protein. In addition, undifferentiated hESCs have *DAZL* expression in mRNA level, but not in protein level, further confirming the translational regulation of *DAZL* in hESCs. Our over expression platform could be used for studying the mechanisms behind the regulation of *DAZL*, which could provide valuable insights for the control of germ cell differentiation.

We suggested that the known functions of NANOS3 and DAZL in mice and other model organism might also be conserved in human germ cell development. In addition, we identified a possible novel function of DAZL in controlling cell migration. It seems that NANOS3 and DAZL may have largely opposing functions; NANOS3 mediates cell migration and suppresses differentiation, while DAZL inhibits cell migration and induces differentiation. We also found indications that NANOS3 may down regulate *DAZL* expression, which would further indicate their opposing roles in germ cell development. Additional *in vitro* and *in vivo* studies are needed to confirm these findings, and the candidate genes identified in our studies provide a good starting point.

Cell transplantation into the germ cell –depleted seminiferous tubules of mice is often used for the functional testing of SSCs, in addition, it has recently been used to assess the germ cell differentiation potential of human PSCs (208-210). We found that after the xenotransplantation, hESCs over expressing DAZL formed spermatogonial-like cell colonies, which had not been achieved with hESCs before. The xenotransplantation assay provides an essential functional testing for germ cell differentiation; however, further optimization is needed in order to eliminate the extensive tumor formation from the transplanted PSCs.

Specifically, the optimal cell number and differentiation state of the cells should be determined.

The development of iPSC technology has enabled the derivation of patient-specific iPSCs that can be used as an *in vitro* model for a disease or possibly in autologous cellular therapies. However, optimization of the reprogramming method is needed in order to produce clinically safe iPSCs. We achieved an efficient integration-free reprogramming of iPSCs using xeno-free and chemically defined culture conditions, which provide critical improvements towards clinically safe iPSCs. For completely xeno-free reprogramming process, however, the derivation and culture conditions for patient fibroblasts must be modified to eliminate the use of animal-derived FBS and trypsin.

We reprogrammed iPSCs from infertile patients diagnosed with KS (47,XXY) for establishing an *in vitro* model to study the effect of the supernumerary X chromosome for germ cell differentiation. The derived iPSCs had XCI, similar to the patient fibroblasts; however, it would be interesting to study the possible genes escaping the XCI and their role in germ cell differentiation. Further studies are needed in order to evaluate the *in vitro* germ cell differentiation potential of the KS iPSCs. The recent advances in converting human PSCs into a naïve state, similar to mouse PSCs, enabled efficient differentiation into germ cells (53, 217). In addition, the naïve human PSCs had both X chromosomes active. Thus, it would be interesting to convert the KS iPSCs into a naïve state, and study their XCI status and germ cell differentiation efficiency.

The recently reported OSCs from adult human ovaries could provide another potential cell source to study germ cells *in vitro*, however, the existence of OSCs is still unclear. There are several shortcomings in the previous report of human OSCs (189). First, the expression of DDX4 in the cell surface, a critical point in the isolation of the cells, was assumed and never tested. Second, the markers used to characterize the putative OSCs, are not specific to germ cells. Third, the formation of haploid oocytes was claimed without showing the indicative polar body formation. And fourth, the reported follicle-enclosed oocytes were shown to be GFP positive by antibody staining and not by native GFP expression. We showed that the DDX4 antibody –based isolation of putative OSCs was not specific for DDX4 expressing cells, and the isolated cells were not functional OSCs. Thus, no evidence for the existence of OSCs was found. More careful studies should be performed with stricter standards before characterizing cells as OSCs.

In conclusion, the recent advances in human PSCs derivation, manipulation and differentiation have enabled new strategies to examine the human germ cell specification and differentiation events, and the genetic requirements of human fertility. In years to come, human PSCs could provide a source for a new form of assisted reproduction.

7 ACKNOWLEDGEMENTS

“It is good to have an end to journey toward; but it is the journey that matters, in the end.”

– Ursula K. Le Guin

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